Antiphospholipid antibodies and the protein C pathway

Christopher Gardiner MSc FIBMS

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration

I, Christopher Gardiner, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The antiphospholipid syndrome (APS) is characterised by the presence of antiphospholipid antibodies (aPA) associated with thrombosis (arterial and venous) and pregnancy morbidity. This thesis has aimed to investigate the frequency of protein C pathway defects in patients with aPA and to study clinical correlates; examine the mechanisms of antiphospholipid interference in the protein C pathway; and to assess activated protein C (APC) resistance in patients with aPA in terms of thrombin generation. Although have I have discovered a high degree of heterogeneity in the phenotype of patients with APS, I have demonstrated APC resistance and increased thrombin generation in the majority of patients with APS. While in some cases, APC resistance is clearly immunoglobulin mediated, it is a multifactorial phenomenon with many confounding variables. My data suggest that immunoglobulin dependent APC resistance may occur through β_2 glycoprotein-I dependent and independent mechanisms. In a detailed study of women with a history of pregnancy morbidity, I have found evidence for an underlying prothrombotic condition, which is due in part to a deficiency of tissue factor pathway inhibitor. This is associated with resistance to APC and increased thrombin generation, both of which may be attenuated through the restoration of normal TFPI levels by low molecular weight heparin.

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List of abbreviations

ACA	Anticardiolipin
aPA	Antiphospholipid antibodies
APC	Activated protein C
APS	Antiphospholipid syndrome
APTT	Activated partial thromboplastin time
β_2GPI	Beta-2 glycoprotein-I
DOPC	di-oleyl-phosphatidylcholine
DOPE	di-oleyl-phosphatidylethanolamine
DOPS	di-oleyl-phosphatidylserine
dRVVT	Dilute Russell's viper venom time
EAPCR	Endogenous activated protein C ratio
ETP	Endogenous thrombin potential
ETP^{+APC}	Endogenous thrombin potential with activated protein C
KCT	Kaolin clotting time
PE	Phosphatidylethanolamine
PT	Prothrombin time
PS	phosphatidylserine
PZ	Protein Z
PZI	Protein Z associated dependent protease inhibitor
SLE	Systemic lupus erythematosus
TAFI	Thrombin activatable fibrinolysis inhibitor (procarboxypeptidase B)
TFPI	Tissue factor pathway inhibitor
VTE	Venous thromboembolism

Chapter 1 Introduction

1.1 History of antiphospholipid antibodies

Antiphospholipid antibodies (aPA) were first recognised in the 1950s, with the description of two unusual phenomena in patients with systemic lupus erythematosus (SLE). In 1952 Conley and Hartman (Conley 1952) described patients with haemorrhagic symptoms due to a non-specific coagulation inhibitor, which acted as an anticoagulant of *in vitro* coagulation tests. In the same year, Moore and Mohr (Moore JE 1952) found that some SLE patients demonstrated persistent false positive serological tests for syphilis and five years later it was found that the 'lupus inhibitor' was frequently associated with false positive syphilis tests (Laurell & Nilsson 1957). Although several publications followed, at the end of the '60s Ethel Bidwell commented "Elucidation of the mode of action of this inhibitor has proved to be extremely difficult, and at the time of writing its aetiology and mode of action remain obscure" (Bidwell 1969).

This *in vitro* coagulation inhibitor was shown to have an association with thrombotic events (Bowie et al. 1963) and was subsequently named the 'lupus anticoagulant' (LA) (Feinstein & Rapaport 1972). Both persistent false positive syphilis tests and LA were also found to be associated with recurrent obstetric problems and thrombocytopenia (Feinstein et al 1972). It later became apparent that antibodies that appeared to bind anionic phospholipids were responsible for both the false positive syphilis tests and LA (Thiagarajan, Pengo, & Shapiro 1986). This allowed the development of more specific tests for anticardiolipin antibodies (aCL) (Harris et al. 1983) and LA (Exner, Rickard, & Kronenberg 1978) (Thiagarajan et al 1986). Following the discovery that the clinically relevant aPA actually recognise phospholipid binding proteins, rather than phospholipids themselves, specific assays for anti β_2 glycoprotein-I antibodies (anti-β2GPI) antibodies were developed (McNeil et al. 1990). Since then, antiphospholipid

antibodies have been described that bind to a variety of phospholipid binding proteins involved in haemostasis (de Groot & Derksen 2005).

1.2 Detection of antiphospholipid antibodies

Three types of test are routinely used for the detection of aPA: lupus anticoagulant tests (eg, dilute Russell's viper venom time, dRVVT); anticardiolipin (aCL); and anti- β2GPI assays.

LA is defined as an immunoglobulin or a group of immunoglobins that interfere with phospholipid (PL) dependent coagulation tests. Paradoxically, LA usually prolongs tests such as the activated partial thromboplastin time (APTT) but is rarely associated with haemorrhage. It is associated with an increased risk of arterial or venous thrombosis, as well as recurrent fetal loss (Hughes, 1993; Triplett, 1995). At the time of writing, the presence of LA is considered to be the strongest risk factor of the aPA tests for thrombosis in (Galli et al. 2003b), although anticardiolipin antibodies may be a stronger independent risk factor for pregnancy morbidity (Robertson et al. 2006)

The APTT is usually prolonged by LA, and fails to correct after patient and normal plasmas are mixed, demonstrating the presence of an inhibitor. This inhibition is usually immediate, although some time dependent inhibitors have been reported. There is wide variation in the sensitivity of different APTT reagents, and the test may be affected by increased levels of fibrinogen and factor VIII, which have the effect of shortening the APTT and may mask weak LA. It is therefore important not to rely solely on the APTT for LA detection, and more sensitive and specific tests are required.

National and international guidelines (Brandt et al. 1995; Greaves et al. 2000) recommend that a sensitive screening test is used, and that a further test is performed for confirmation. A large number of tests have been suggested for LA screening and confirmation (Figure 1), which detect interference with different parts of the coagulation

pathway. It is generally recommended that two tests should be selected which operate by different coagulation mechanisms or reactions. The tests used should demonstrate the presence of an inhibitor and phospholipid dependence. The latter is usually achieved using either high concentrations of phospholipid, or using washed normal platelets that have been activated or lysed, so that they express LA bypassing activity.

Figure 1. Detection of lupus anticoagulant antibodies by in vitro coagulation tests: APTT activated partial thromboplastin time CSCT Colloidal silica clotting time, KCT kaolin clotting time, dPT dilute thromboplastin time (Levine, Branch, & Rauch 2002)

The most widely used test in the UK is the dRVVT. This test utilises the venom of Russell's Viper (*Daboia russelii*), which contains an enzyme that directly activates factor X (Thiagarajan et al 1986). In the presence of factor V, the resulting factor Xa cleaves prothrombin to thrombin in a reaction dependent on the presence of calcium

ions and suitable concentrations of coagulant active phospholipid (the prothrombinase complex). Fibrin is then formed, and clot detection is the end-point of the test. The venom is diluted to make the test sensitive to any small prolongations of the clotting time, and the phospholipid is diluted to make the test sensitive to any interference with phospholipid dependent coagulation reactions. The dRVVT should be performed in conjunction with a confirmatory step to assure specificity. This may be achieved by substituting the dilute phospholipid reagent with a high concentration of phospholipid, or by using washed, lysed platelets (the platelet neutralisation procedure). The mechanism by which the platelets neutralise or by-pass LA is unknown, but it is possible that they protect the prothrombinase complex from inhibition, either by causing steric hindrance of the immunoglobulins, or because the reaction kinetics favour prothrombinase.

The kaolin clotting time (KCT) was previously widely used for the detection of LA (Exner et al 1978), but suffers from poor sensitivity and specificity (Jennings et al. 1997) as it is more susceptible to variation due to poor sample quality.

Other tests such as the Textarin/Ecarin ratio (Triplett et al. 1993), the Taipan venom time (Rooney et al. 1994) and the dilute thromboplastin test (Schleider et al. 1976) are used principally as additional confirmatory tests or for use with plasma from patients receiving oral anticoagulants, and will not be discussed further.

Anticardiolipin and anti- β_2GPI antibodies (McNeil et al 1990) are both detected using solid phase enzyme linked immunosorbent assays (ELISA). In the aCL ELISA, microtitre plates are coated with cardiolipin and non-specific binding of diluted sera is eliminated by blocking of plates with 10% fetal calf serum (Loizou et al. 1985). Diluted patient sera are then added to the plates and antibody binding is detected through the use of enzyme-conjugated antisera. Galli et al. (1990) noted that highly purified antibodies did not bind cardiolipin in assay systems in which bovine serum was not added, and that

a protein cofactor was required for antibody binding. This cofactor was subsequently confirmed as β_2GPI or apolipoprotein H (McNeil et al 1990). Consequently, anti-β_2GPI antibodies are now measured using an ELISA in which an ELISA plate is coated with β_2GPI, and binding of antibodies to the immobilised protein in an antiphospholipid independent manner is measured (Martinuzzo, Forastiero, & Carreras 1995; McNally et al. 1995a). This is made possible through the binding of β2GPI to the charged surface of an irradiated plate, which alters the confirmation of the β2GPI molecule thus exposing the epitope to which the anti- β2GPI antibodies bind (Matsuura et al. 1994). It is thought that this process mimics the effect of anti-phospholipid binding. Our group at UCL demonstrated that anti- β2GPI discriminated between anticardiolipin antibodies associated with infection and those associated with an increased risk of thrombosis (McNally et al 1995a; McNally et al. 1995b).

Although in theory, the aCL and anti- β2GPI ELISA detect the same antibodies, it is not uncommon to encounter serum from patients with aPA that bind cardiolipin but not β_2GPI, and vice versa. This may be partly explained by the fact that some anti- β_2GPI recognise human β_2GPI, but not bovine β2GPI in aCL assays. The bovine serum in aCL assays provides antigenic targets other than β_2GPI. One would expect anti- β2GPI assays to show better agreement between centres, as only one well-defined antigen is measured, but collaborative studies have shown that inter-laboratory agreement remains poor Antigen density, protein damage through the use of perchloric acid, proteolysis of β_2GPI and β_2GPI isoforms may all contribute to poor standardisation (Reber et al. 2005). Therein lies the problem, in that we do not fully understand what solid phase antibody detection tests are measuring. The proponents of anti- β2GPI point to the increased specificity of these antibodies (de Groot et al 2005; Sheng, Kandiah, & Krilis 1998) others have suggested that their measurement adds no additional information to the diagnosis of APS (Previtali, Barbui, & Galli 2002). At the scientific and

standardisation committee (SSC) of the International Society for Thrombosis and Haemostasis (ISTH) at Boston in 2002, it was suggested that the aCL test would be better replaced with a direct test of anti- β2GPI. This has since been challenged by our group (Nash et al. 2004), who showed that omission of aCL would lead to a failure to diagnose APS in over 25% of patients.

Clearly problems remain with the laboratory diagnosis of the APS. The antibody specificity, concentration and avidity are extremely variable among APS patients and not all of the known target antigens are represented by the antibodies detected by the routine tests. Indeed some authorities believe that antibodies measured in aPA laboratory tests are epiphenomenona, rather than pathogenic in their own right.

1.3 The antiphospholipid syndrome

The association of aPA with thrombosis, stroke and/or fetal loss is termed 'Antiphospholipid Syndrome (APS) (Hughes 1983). It is now known that many of patients with these clinical symptoms and laboratory findings do not suffer from SLE or related autoimmune diseases (e.g., Sjögren's and scleroderma) and these patients are said to have Primary Antiphospholipid Syndrome (PAPS). Other clinical associations include neurological disorders and immune mediated thrombocytopenia.

1.3.1 Thrombosis

Venous thrombosis occurs in the western world with a frequency of approximately 1 per 1000 individuals per year. It is associated with a high degree of morbidity and, if pulmonary embolism develops, it may be fatal. Venous thrombosis is a multifactorial process, which is generally the result of one or more inherited or acquired risk factors, eg increasing age, malignancy, orthopaedic surgery, obesity, immobilisation, puerperium, the use of combined oral contraceptives (COC) and of course antiphospholipid antibodies. In some instances, there appears to be a strong hereditary

tendency to thrombosis, and this is known as heritable thrombophilia (Greaves et al. 2001). APS is considered by many to be the most important acquired thrombophilic condition, with strong associations between the presence of aPA and thrombosis both in patients with SLE (Wahl et al. 1997) and without underlying autoimmune disorders (Wahl et al. 1998). In patients affected by thrombophilia, the first incidence of thrombosis is commonly seen at a relatively early age and often without any obvious precipitating cause. It has been reported that at least one genetic defect predisposing to thrombophilia can be found in 70% of families with thrombophilia (Bertina 1997). Many of these hereditary risk factors are quite common and, when found in isolation, are associated with only a slightly increased incidence of thrombosis. However, when co-inherited with other defects, there is a synergistic effect, resulting in a much higher occurrence of thrombotic disorders (Rosendaal 1997).

The association between aPA and thrombosis had long been recognised (Bowie et al 1963). LA followed by medium/high titre IgG aCL are most strongly associated with venous thrombosis (Galli et al 2003b). Anti-prothrombin antibodies and anti- β2GPI also demonstrate an association with thrombosis, but there is little data and more studies are required (Galli et al. 2003a).

The aetiology of arterial thrombosis is more complex than that of venous thrombosis, but tends to associated with abnormalities of the vessel wall, disordered lipid metabolism and excessive platelet activation rather than the heritable thrombophilic defects associated with venous thrombosis. However, APS is an important contributing factor for arterial thrombosis, particularly in younger patients.

1.3.2 Pregnancy morbidity

The association of a circulating anticoagulant and pregnancy loss was first described by Nilsson (Nilsson et al. 1975), who reported the presence of "antithromboplastin" in a woman who had three interuterine deaths characterised by placental infarction. It is

known that in pregnant women with SLE, anticardiolipin is a predictor of fetal loss (Lockshin et al. 1985) and that pregnancy losses tend to occur later than in SLE patients without anticardiolipin (Loizou et al. 1988).

There is considerable controversy over which tests are most useful in predicting pregnancy morbidity. Creagh *et al* (1991) reported that LA and IgG aCL are risk factors for recurrent pregnancy loss, while anti- β_2GPI antibodies have been shown by others to be most strongly associated with recurrent pregnancy loss (Falcon et al. 1997; Forastiero et al. 1997). Our group have shown that the presence of anti-prothrombin antibodies are linked with fetal loss (Donohoe et al. 2001), but this has been disputed by others (Falcon et al 1997; Forastiero et al 1997) and may depend upon the methodology used as well as the selection of the clinical cohort. Antibodies against factor XII (Jones et al. 2001) and annexin V (Rand et al. 1997) have also been implicated in the pathology of aPA associated pregnancy morbidity

In one of the few prospective studies performed, Rai *et al* (1995) reported a fetal loss rate of 90% in untreated pregnancies of women with recurrent miscarriage and antiphospholipid antibodies. At present, the mainstay of treatment for high-risk pregnancies is aspirin and/or low molecular weight heparin (LMWH) (Brenner et al. 2005; Rai et al. 1997) which improves the live birth rate. However, even these treated pregnancies have a high rate of complications (Backos et al. 1999; Brenner et al 2005; Lima et al. 1996) and the effectiveness of LMWH has been disputed (Farquharson, Quenby, & Greaves 2002).

1.3.3 Other conditions associated with antiphospholipid antibodies

The antiphospholipid syndrome is frequently associated with other autoimmune conditions, and although the differentiation between primary and secondary APS may not be useful in the treatment of the thrombotic or obstetric complications, it may aid the diagnosis. Thrombocytopenia, livedo reticularis, migraine, valvar heart disease and

cognitive dysfunction are all commonly reported, although the causal link with aPA is often tentative at best.

A rare, but severe complication is catastrophic antiphospholipid syndrome, which is an acute condition cause by intravascular thrombosis at multiple sites, affecting several organs. It is frequently associated with disseminated intravascular coagulation, which is not seen in APS, and is often fatal.

1.4 Normal haemostasis

The haemostatic system has two principal functions: To keep blood fluid in normal circulation; and to limit the loss of blood when blood vessels become damaged. Normal haemostasis involves complex interactions between procoagulant, anticoagulant and fibrinolytic plasma proteins, platelets, leukocytes and the vascular endothelium.

The generation of thrombin is the central event in blood coagulation: Insufficient thrombin leads to an increased risk of bleeding, while unregulated thrombin generation predisposes to thrombosis. While the prothrombin time and activated partial thromboplastin time may be useful tests in identifying bleeding risk, they are of little value in predicting thrombotic risk, as they do not reflect the overall thrombin generation. In order for blood to clot, it is only necessary for a small proportion of the available prothrombin to be converted to thrombin, with >95% of thrombin formation occurring after this point (Brummel et al. 2002). The endogenous thrombin potential (ETP), i.e. the area under the thrombin generation curve, better represents this.

The laboratory investigation of thrombophilia, in particular, has become increasingly reductionist in approach over the last three decades, i.e. the amount, the activity and mutations or polymorphisms of individual haemostatic factors are measured in order to predict thrombotic risk. The clinical utility of some of these measurements, however, is questionable in predicting thrombotic risk and many now feel that a global assessment

of the capacity of a patient's blood to generate thrombin (ETP) may be more useful in this setting (Baglin 2005).

Thrombin generation is now a widely used research tool, with applications in the investigation of bleeding and thrombotic disorders. A method for thrombin generation was first described by MacFarlane and Biggs (1953), who observed, "Experimental procedures have become increasingly complex and artificial. These techniques have yielded information of great importance, but they must have a limited application, since they are far removed from the natural process of clotting". It was not until 1993 when Hemker et al (1993) described a method for the continuous registration of thrombin generation, that it became a practical technique even for dedicated research laboratories. This technique, while useful for small numbers of samples, was technically demanding and time consuming. The development of slow acting thrombin substrates (Wielders et al. 1997) allowed the semi-automated measurement of ETP in defibrinated plasma using a centrifugal analyser.

Although the components of the haemostatic system are intertwined, for practical reasons it is more convenient to describe them separately, although we should never forget how closely the various components are linked.

1.4.1 Primary haemostasis

Primary haemostasis is characterized by vascular contraction, platelet adhesion and formation of a soft aggregate plug. Immediately after a blood vessel has been cut or ruptured, the trauma to the vessel wall itself causes the vessel to contract (vasoconstriction, vascular spasm), instantaneously reducing blood flow from the vessel rupture. The local vascular spasm can last for many minutes or even hours, during which time the process of platelet plug formation and blood coagulation can take place. This is followed by platelet adhesion to von Willebrand factor(vWf) secreted from the subendothelium into the subendothelial matrix. Glycoproteins on the platelet surface

adhere to the von Willebrand factor(vWf) forming an unstable platelet plug that is strengthened by contact with collagen. Collagen-activated platelets form pseudopods, which stretch out to cover the exposed subendothelium and bridge exposed collagen fibres. The collagen-activated platelet exposes receptors that bind circulating fibrinogen to their surfaces and the fibrinogen recruits more platelets. The activated platelets play a major role in thrombin generation, which consolidates the platelet plug to form a clot.

1.4.2 The role of the endothelium

Endothelial integrity is essential for the function of blood vessels and maintenance of a non-thrombotic state. Under normal conditions, blood flows unimpeded through the circulatory system. However, following injury, local vasoconstriction occurs instantaneously in order to reduce loss of blood from the damaged vessel. Thrombin acts as a pivot in the maintenance of the haemostatic balance; the vascular endothelial cell in particular limits the generation of thrombin by localisation of anticoagulant processes on its luminal membrane. The endothelial cell synthesises key molecules in this process and also binds exogenously derived molecules, as well as releasing proteins of the fibrinolysis cascade. The thrombo-resistance of the luminal surface is further regulated by lipoxygenase and cyclo-oxygenase metabolites of unsaturated fatty acids synthesised by the endothelial cell. In response to trauma, inflammatory reactions, normal wound healing and in association with a variety of disease states, the anticoagulant and fibrinolytic mechanisms are down-regulated and the procoagulant and thrombotic mechanisms predominate with resultant generation of thrombin, fibrin clot formation and subsequent platelet adhesion and aggregation. Pro-inflammatory cytokines down-regulate the fibrinolytic and activated protein C pathways as well as inducing synthesis of specific procoagulant and prothrombotic mediators by platelets and leukocytes as well as endothelium

1.4.3 Coagulation

The cascade or waterfall hypothesis of coagulation, developed in the 1950s and 1960s, depicts the coagulation system as a series of proteolytic reactions (Figure 2). It helped us to understand the interactions between the various coagulation factors and also explained the importance of anionic phospholipids and calcium. The cascade model correlates very well with the PT and APTT screening tests, and for this reason, it is still widely taught today. However, it has several serious limitations, in that it cannot be used to explain why factors VIII and IX deficiencies cause haemophilia, yet deficiencies in the contact factors are not associated with a bleeding diathesis. When Osterud (Osterud & Rapaport 1977) demonstrated that factor IX could be directly activated by the factor VIIa/tissue factor complex, it became clear that the cascade model was an over simplification of the coagulation process.

Central to coagulation are the vitamin K dependent proteins which include the coagulation factors II, VII, IX, X and the anticoagulant proteins C, S and Z. These proteins all contain a glutamic acid-rich γ-carboxyglutamic acid or "gla domain" which is the result of gamma carboxylation, and is required for the proteins to bind to phospholipid and calcium. Gamma carboxylation is a vitamin K dependent process and the inhibition of this process is the basis of oral anticoagulants such as warfarin. Without gamma carboxylation these proteins are largely inactive and this is the basis of vitamin K antagonist anticoagulants such as warfarin. The gla domain allows the correct orientation and alignment of procoagulant and anticoagulant proteins on negatively charged antiphospholipid surfaces.

Figure 2 The cascade model of coagulation
(from Hoffman and Monroe (Hoffman & Monroe, III 2001)

The realisation that cells play a central role in haemostasis, rather than simply providing an anionic phospholipid surface (Hoffman et al 2001; Roberts, Hoffman, & Monroe 2006) has revolutionised the field. The prevailing view for many years was that thrombosis was simply haemostasis occurring in the wrong place, whereas it is now generally accepted that normal haemostasis and thrombosis are very different processes. Key to the cellular theory of haemostasis is the idea that different stages of coagulation occur in different sites. The three stages are, initiation, propagation and amplification.

Initiation of thrombin generation occurs on tissue factor bearing cells. These may be extravascular cells, which constitutively express TF. Alternatively, in some pathological states, monocytes and endothelial cells, may express inducible TF due to stimulation by endotoxin, inflammatory cytokines or aPA. Factor VIIa/TF dependent generation of factors IXa, FXa and FVa are generally held in check by tissue factor pathway inhibitor (TFPI) and to a lesser extent, protein Z-protein Z-dependent protease inhibitor complex

(PZ/PZI). It has been suggested that formation of the prothrombinase complex is a threshold event that only occurs when there is sufficient TF, and hence extrinsic tenase complex, to overcome TFPI inhibition (van 't Veer & Mann 1997b).

The propagation stage occurs on the surface of activated platelets, and generates factors IXa, Xa, Va and trace amounts of thrombin. Antithrombin readily inhibits free factor Xa and thrombin, but factor IXa less so. Factor IXa is thus able to move to other sites.

The amplification phase occurs on several activated platelets. Feedback through thrombin activation of factor XI leads to an amplification loop, hence the importance of factors VIII and IX. The protein C pathway does not function well on the surface of activated platelets, but when the thrombin leaves the vicinity of the haemostatic plug and reaches an area of intact endothelium it is bound to thrombomodulin with high affinity. The thrombin-thrombomodulin complex has no procoagulant activity, but activates protein C to activated protein C. This in turn inactivates factors Va and VIIIa in a protein S catalysed reaction, thus limiting coagulation to the site of injury.

Such is the complexity of the coagulation system, that it may be best understood as a series of key complexes.

The extrinsic tenase complex forms on the tissue factor-bearing cell (Figure 3). Factor Xa is generated, and small amounts of thrombin are produced, which activate platelets and release active factor VIII from vWf. It also generates factor IXa which is able to move to other sites where the propagation phase occurs. This complex is controlled by TFPI.

Figure 3 Extrinsic tenase complex from Mann et al (2006)

Factor IXa forms a complex with factor VIIIa and factor Xa (Figure 4). This complex is many times more efficient than the extrinsic tenase complex, and generates many molecules of Xa on activated platelets. This is the propagation phase.

Figure 4 intrinsic tenase Mann et al (2006)

The prothrombinase complex also forms on the activated platelet (Figure 5). Factor Va acts as a cofactor for factor Xa and generates large amounts of thrombin. This thrombin may feed back through factor XI to cause further amplification.

Figure 5 prothrombinase complex Mann et al (2006)

When thrombin reaches in tact endothelium, it is tightly bound by thrombomodulin and alters it substrate specificity to protein C (Figure 6). The APC formed controls the formation of thrombin through the inactivation of factors Va and VIIIa.

Figure 6 Protein C-ase Mann et al (2006)

1.4.4 Inhibitors of coagulation

Most cases of thrombophilia, involving venous disease, appear to involve either inappropriate thrombin generation or decreased thrombin inhibition. There are four levels of control of thrombin generation:

- proteins such as β_2 glycoprotein-I and annexin V limit the availability of negatively charged phospholipid surfaces, which support most coagulation reactions
- tissue factor pathway inhibitor (TFPI) controls the availability of the active tissue factor/factor VIIa complex, major determinants of the amount of thrombin generated
- the protein C system inactivates the activated cofactors FVIIIa and FVa, which amplify factor Xa and thrombin generation.
- a range of serine protease inhibitors, of which antithrombin is the most important, limit the activity of activated clotting factors (eg thrombin, factor Xa)

As with most aspects of haemostasis, this is an over-simplification. It is now known that TFPI and the protein C pathway act in synergy (van 't Veer et al. 1997a). In purified systems, the presence of normal plasma levels of TFPI, protein C and protein S, and 1nM thrombomodulin, is sufficient to eliminate all prothrombinase activity initiated by 1.25 pM factor VIIa.TF. Thus, TFPI and the protein C pathway combine to create a minimal inhibitory potential required to shut down tissue factor-initiated thrombin generation. Defects in these control mechanisms may result in predisposition to venous thrombosis proportional to the severity of the deficiency and the importance of the control mechanism involved. Hence, a homozygous deficiency of antithrombin, which is the major plasma inhibitor of thrombin, is thought to be incompatible with life,

whereas homozygous β2–glycoprotein-1 deficiency does not appear to be associated with an increased risk of thrombosis.

1.4.5 The protein C system

The protein C anticoagulant pathway serves as a major system for controlling haemostasis and limiting inflammatory responses. The pathway involves thrombin, thrombomodulin, endothelial cell protein C receptor (EPCR), protein C, and protein S, but, so far as haemostasis is concerned, APC is the main effector molecule (Figure 7). Homozygous deficiencies of protein S and C cause purpura fulminans and require treatment from birth. Thrombomodulin and EPCR gene knockouts are lethal in mice, and the homozygous deficient state has not been reported in humans, underlying the importance of the protein C system. Disturbances in the protein C pathway are strongly implicated in the aetiology of thrombosis, stroke and recurrent miscarriage. The majority of familial thrombophilia defects discovered so far reside in the protein C pathway (Rosendaal et al, 1997); genetic defects linked to thrombophilic states have now been described in protein C, protein S, EPCR, thrombomodulin and factor V.

Figure 7 The role of protein C in regulating coagulation From Esmon (Esmon 2003)

Current laboratory diagnosis of defects in the protein C system relies on the following types of specific assay.

- Clotting tests based on the prolongation of a clotting time by APC, which may be formed by the activation of endogenous protein C using Protac™ (an extract from Agkistrodon Contortrix venom) or the addition of exogenous APC.
- Amidolytic assays that utilise the ability of APC to cleave synthetic peptide substrates, producing p-Nitroaniline, which can be, measured photocolorimetrically.
- Immunological assays that use specific monoclonal antibodies to recognise proteins. The most commonly used assays are ELISA, although automated latex agglutination assays for free protein S are now widely available.

1.4.5.1 Protein C

Protein C is a vitamin K-dependent glycoprotein first described in 1976 (Esmon, Stenflo, & Suttie 1976). Unlike the other vitamin K dependent zymogens known at that time, protein C is not required for blood coagulation, and was subsequently found to inhibit thrombin formation (Comp & Esmon 1979; Dahlback & Stenflo 1980). We now know that this occurs through the inactivation of factor Va (Walker 1980) and factor VIIIa. Hereditary protein C deficiency was first described in a three Dutch families with a high incidence of venous thromboembolism (Broekmans, Veltkamp, & Bertina 1983). It has been estimated that approximately 3% of patients presenting with their first episode of deep vein thrombosis (DVT) and 6% of individuals with familial thrombophilia are protein C deficient. Large studies of blood donors show that the prevalence of heterozygous protein C deficiency is approximately 1 in 400, yet most of these subjects are asymptomatic, suggesting that an additional risk factor for thrombosis is required in heterozygous deficiency (Rosendaal et al, 1997). However, homozygous

protein C deficiency is a severe disease, which usually presents in the neonate as purpura fulminans or disseminated intravascular coagulation. Using qualitative and quantitative assays it is possible to identify two main types of protein C deficiency. Type I is the most common and refers to a decrease in antigen with a concomitant decrease in activity; whereas in type II, antigenic levels are normal but a dysfunctional protein results in reduced activity.

The activated form of protein C (APC) has both anticoagulant and anti-inflammatory functions and has been shown to reduce mortality in patients with sepsis (Bernard et al. 2001). The recombinant human form (rhAPC, Drotrecogin Alfa, Eli Lilly) is now widely used in the treatment of systemic inflammatory response syndrome in sepsis.

1.4.5.2 Protein S

Protein S is a vitamin K-dependent plasma glycoprotein and was discovered three years after protein C (DiScipio & Davie 1979). In the circulation, Protein S exists in two forms: a free form and a complex form bound to C4b binding protein. It is rare amongst vitamin K dependent glycoproteins, in that it has no serine protease activity, but acts as a cofactor for other enzymes. Protein S has several functions, and it is convenient to divide these into APC-dependent and independent functions.

Protein S forms a complex with APC on negatively charged phospholipid and potentiates the anticoagulant effect of APC on factor Va and factor VIIIa (Walker 1980). C4b-binding protein inhibits the factor V-dependent but not the factor V-independent cofactor activity of protein S in the activated protein C-mediated inactivation of factor VIIIa (van de Poel, Meijers, & Bouma 2001).

It is known that protein S has an inhibitor effect of factor Xa (Heeb et al. 1994), independent of APC. Protein S also recognises apoptotic cells and this allows the C4b-

binding protein-protein S complex to prevent secondary necrosis through complement activation (Rezende, Simmonds, & Lane 2004).

A strong association between protein S deficiency and familial thrombophilia exists with a similar clinical presentation to that of protein C deficiency. There are no reliable estimates for the prevalence of protein S deficiency in a normal population, but estimates for patients presenting with first DVT and familial thrombophilia are approximately 1.4% and 7.2% respectively (Rosendaal 1997). Free and total protein S levels are known to fluctuate over time, and to be strongly influenced by acute phase reactions, age, sex and hormonal status (Liberti, Bertina, & Rosendaal 1999).

1.4.5.3 Thrombomodulin

Thrombomodulin, an integral membrane protein, is expressed on the surface of vascular endothelial cells and the syncytiotrophoblast. It binds thrombin in a high affinty1:1 stoichiometric non-covalent complex, which alters the specificity of thrombin so that it preferentially cleaves a small peptide bond in the protein C heavy chain, to form the active serine protease: APC. Once bound to thrombomodulin, the substrate specificity of thrombin is redirected from procoagulant to anticoagulant reactions. The thrombomodulin-bound thrombin activates protein C and thrombin-activatable fibrinolysis inhibitor (TAFI) at a rate ~1000 greater than thrombin alone (Lane, Philippou, & Huntington 2005). Although thrombomodulin polymorphisms and defects are rare in patients with thrombosis, antibodies to thrombomodulin have been described in APS and may inhibit protein C activation (Carson et al. 2000; Oosting et al. 1993b). Mutations in the thrombomodulin gene are associated with an increased risk of late fetal loss (Franchi et al. 2001) and mouse knockout models have shown that thrombomodulin is essential for fetal development (Isermann et al. 2003).

1.4.5.4 Endothelial protein C receptor

The endothelial protein C receptor (ECPR) is also a transmembrane protein, found primarily on the endothelium of large vessels and trophoblasts (Esmon 2006). EPCR binds protein C and facilitates activation by the thrombin-thrombomodulin complex, increasing the rate of APC formation twenty fold (Stearns-Kurosawa et al. 1996)

EPCR also binds activated protein C (APC) and inhibits the anticoagulant activity of APC, but not its anti-inflammatory functions (Regan et al. 1996). ECPR is essential for embryonic viability (Li et al. 2005) (Gu et al. 2002). Furthermore, a polymorphism in the ECPR resulting in increased shedding of the receptor is associated with increased risk of thrombosis (Medina et al. 2004) and pregnancy loss in humans (Lavigne-Lissalde et al. 2005). Antibodies against EPCR in APS have been reported to be a risk factor for fetal death (Hurtado et al. 2004).

1.4.5.5 Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is a Kunitz-type serine proteinase inhibitor that down-regulates tissue factor/factor VIIa-initiated blood coagulation via a two-step negative feedback mechanism, through formation of a bimolecular FXa/TFPI complex. This complex subsequently interacts with TF/FVIIa, yielding an inactive quaternary complex of Xa, TFPI, and factor VIIa and tissue factor, thus terminating TF/FVIIa-catalysed FX activation. TFPI contributes significantly to the inhibition of Xa *in vitro* (van 't Veer et al 1997b), despite being present at concentrations of only 2.5nM. The association of low levels of plasma TFPI with venous thrombosis (Dahm et al. 2003), suggests that TFPI is also important *in vivo* and indeed, homozygous TFPI deficiency causes a lethal phenotype.

Although not considered a part of the protein C system, TFPI may act in synergy with APC, in purified systems (van 't Veer et al 1997a). TFPI is associated with vascular

endothelium and is released into the bloodstream by heparin (Sandset, Abildgaard, & Larsen 1988). Both TFPI (Mast et al. 2002) and a related molecule tissue factor pathway inhibitor type-2 (TFPI-2 also known as placental protein 5) are associated with placenta (Udagawa et al. 2002).

1.4.5.5 Resistance to activated protein C

The most common inherited cause of thrombophilia is activated protein C resistance (APCR). This defect is usually associated (>80%) with a point mutation in the factor V gene resulting in an Arg506Gln substitution which renders the mutant protein, factor V Leiden (FVL or R506Q), resistant to cleavage by APC (Bertina 1997). The factor V Leiden defect occurs in approximately 2 – 16 % of Caucasian populations although it is extremely rare in Asians, Arabs and Africans. Factor V Leiden is a fairly common defect, which may be readily confirmed by the identification of a single polymorphism. These features have allowed studies involving large numbers of affected individuals, previously impossible with rarer mutations. Consequently, much more is known about the increased risk of thrombosis associated with factor V Leiden and interactions with other thrombogenic risk factors, than for other defects. Heterozygous factor V Leiden is associated with a 3 to 7 fold increased risk of thrombosis, whereas the homozygous form has an 80-fold increase. When factor V Leiden is co-inherited with other defects, such as protein C, deficiency, protein S deficiency or the prothrombin gene mutation 20210A, there is a synergistic effect resulting in an increased risk greatly exceeding the sum of the individual risk factors. Recent work has identified three other polymorphisms in the factor V gene that may be thrombotic risk factors. Factor V Cambridge (Arg306Thr) and homozygosity for the HR2 haplotype for the factor V gene both cause APCR (de Visser et al. 2000), whereas the Arg306Gly mutation first discovered in Hong Kong Chinese does not appear to be associated with APCR (Norstrom, Thorelli, & Dahlback 2002). Several conditions are associated with acquired

APCR that may also be a risk factors for thrombosis including lupus anticoagulant, pregnancy, combined oral contraceptive use and elevated factor VIII (Rosendaal 1997).

1.4.5.6 Antithrombin

Antithrombin, previously known as antithrombin III, is a serpin that inactivates a number of enzymes from the coagulation system, namely thrombin, factor Xa, factor IXa, Factor XIa, and Factor XIIa. The rate of its reaction with these molecules is greatly enhanced by the presence of heparin. This is achieved through two functional domains, a heparin binding site and a reactive centre, that complexes and inactivates the proteinase (Lane et al. 1996). The heterozygous inherited state and acquired deficiencies of antithrombin result in a hypercoagulable state. The homozygous deficient state has not been described and is thought to be incompatible with life.

1.4.5.6 Fibrinolysis

Plasmin is the major fibrinolytic protease. Plasminogen, a circulating plasma zymogen, can be converted to plasmin by both tissue plasminogen activator (tPA) and urokinase (uPA). Fibrin, the major plasmin substrate, regulates its own degradation by binding both plasminogen and tPA on its surface, thereby localizing and enhancing plasmin generation. While tPA is a weak activator of plasminogen in the absence of fibrin, its catalytic efficiency for plasminogen activation is enhanced in the presence of fibrin. Plasmin cleaves fibrin, generating soluble degradation products (Cesarman-Maus & Hajjar 2005).

tPA and urokinase are themselves inhibited by plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2 (PAI-1 and PAI-2). Plasmin stimulates its own production through the cleavage of single chain tPA and urokinase to more

active two-chain polypeptides. Alpha 2-antiplasmin and alpha 2-macroglobulin inactivate plasmin. Plasmin activity is also reduced by thrombin-activatable fibrinolysis

inhibitor (TAFI), which removes the carboxy-terminal Lys residues of fibrin, which modifies fibrin to make a less potent cofactor for the tPA-mediated plasminogen activation. This has the effect of stabilising the fibrin thrombus and represents a regulatory connection between coagulation and fibrinolytic pathways.

Defective fibrinolysis has been described in APS and several mechanisms have been proposed (Keeling et al. 1991; Patterson et al. 2006; Takeuchi et al. 2002; Yang et al. 2004).

1.4.6. The role of phospholipids in haemostasis

The central role of the membrane surface in the reactions of the coagulation cascade has been well known for many years (Mann et al. 1990). A negative charge on the membrane is recognized as necessary for the binding of the vitamin K-dependent enzymes and substrates through their N-terminal Gla domains. Through this binding, the local concentrations of the proteins are markedly increased, augmenting activation rates. Binding may also induce conformational changes in the proteins, aligning substrate cleavage sites with the active site of the enzyme (Mutucumarana et al. 1992). The nature of the phospholipid head group was known to play a role, and phosphatidylserine (PS) has been considered the most important (Pei, Powers, & Lentz 1993). For many years, it was believed the reactions of the coagulation cascade shared similar requirements for the membrane surface. However, most experiments were performed with the prothrombin-activating complex, prothrombinase, with a tendency to generalise to the other complexes. More recently, it has become apparent that the presence of phosphatidylethanolamine (PE) potently enhanced the rate of inactivation of factor Va by the activated protein C (APC) complex (Smirnov & Esmon 1994) and the anticoagulant activity in plasma while having little to no effect on the prothrombinase reaction when the PS concentration was optimal. (Pecheniuk 2003)

The role of oxidation in disease has gained considerable attention in the last few years. Oxidation is believed to play a key role in the pathogenesis of many inflammatory diseases including atherosclerosis, reperfusion injury, and autoimmune diseases. Oxidized LDL plays a major role in the initiation and propagation of the atherosclerotic plaque (Ross 1999). It can also lead to the activation of endothelium and platelets. Endothelial cell activation can then lead to the elaboration of other inflammatory mediators which themselves exacerbate coagulation. Oxidized LDL can also be immunogenic and lead to the development of antibodies that cross-react with cardiolipin or the surface of endothelial cells. In addition, apoptotic cells with negatively charged surfaces can be immunogenic and lead to the development of aPA. These observations led Horrko *et al*(1996) to determine that many aPA are directed toward epitopes of oxidized phospholipids and or adducts of oxidized phospholipid and β2–glycoprotein I.

More recently, Safa et al (2001) have shown that oxidation of natural phospholipids increases the ability of the membranes to support the function of APC, and potentiated the APC cofactor activity in of protein S in factor Va inactivation, without significantly altering the ability to support thrombin generation. Cardiolipin also enhances the protein C pathway anticoagulant activity (Fernandez et al. 2000) and has been shown to be a normal component of low density lipoprotein in human plasma (Deguchi et al. 2000)

Oxidation has long been recognized as having an effect in the *in vitro* coagulation assays (Barrowcliffe, Stocks, & Gray 1982), usually observed as a change in clotting time with the age of the phospholipid preparation. It is also known that oxidised lipid have a profound effect on the protein C pathway (Safa et al. 2001; Safa, Esmon, & Esmon 2005).

1.5 Proposed mechanisms for pathogenesis of antiphospholipid antibodies

Many of the autoantibodies associated with APS are directed against phospholipid-binding plasma proteins, such as beta2-GPI and prothrombin, or phospholipid-protein complexes. The mechanisms by which aPL cause thrombosis are not completely understood. There is no unique mechanism able to explain all symptoms associated with the presence of aPL. A variety of mechanisms have been proposed to explain the clinical phenomena, including abnormal activation and function of protein C, activated platelets, reduced prostacyclin production, disordered fibrinolysis, and inhibition or potentiation of the actions of β2-Glycoprotein I. As with other thrombotic disorders, the pathogenesis of APS is likely to be multifactorial.

1.5.1 Endothelial activation

Several different theories have been proposed for the ability of aPA to induce endothelial activation. It has been shown that aPA react with directly with endothelium (Hill et al. 1995; Hill et al. 1998) and that this interferes with fibrinolysis on the cell surface. (Patterson et al 2006). aPA have been shown to induce tissue factor on endothelial cells (Branch & Rodgers 1993). It has been proposed that this occurs through p38 MAPK in the up-regulation of tissue factor NF-kappa B (Vega-Ostertag et al. 2005) and that this process may be β_2GPI dependent (Kornberg et al. 2000). Furthermore, endothelial activation may also lead to the release of tissue factor bearing endothelial microparticles (Combes et al. 1999). Inhibition of prostacyclin release by endothelial binding aPA has also been reported (Lindsey et al. 1994). Aspirin has been shown to inhibit aPA dependent endothelial activation (Dunoyer-Geindre et al. 2004).

1.5.2 Monocyte activation

Over-expression of TF has been also shown in monocytes in vitro and ex vivo. TF is the major initiator of coagulation in vivo; thus, its dysregulation may be one of the most important contributors to thrombosis. It has been reported that some aPA may induce tissue factor on monocytes (Cuadrado et al. 1997) enhance thrombin generation on tissue factor binding cells (Hoffman, Monroe, & Roubey 2000).It has recently been shown that aPA induce tissue factor expression through the simultaneous activation of NF-kappa B/Rel proteins (Lopez-Pedrera et al. 2006)

1.5.3 Platelet activation

Effects of aPL upon platelets are not completely elucidated. aPL bind anionic phospholipid but they are normally in the inner side of cell membranes. When platelets are activated, anionic phospholipids are exposed. There is evidence showing that activated platelets are present in aPL-positive patients (Joseph et al. 1998; Joseph et al. 2001). This was supported by Nojima *et al* (Nojima et al. 2004) who reported that aPA enhance ADP induced platelet activation and may predispose to stroke. A role for anti-β_2-GP I antibodies has been suggested, in which anti-β_2-GP I/ β_2-GP I complexes activate platelets in a dysregulated manner via glycoprotein Ib-IX-V.

1.5.4 The role of anti β_2 Glycoprotein-I antibodies

In recent years there has been a great deal of interest in the role of anti-β_2-GP I antibodies. At first sight, it is difficult to see why antibodies against β_2-GP I should be pathogenic at all. Although, β_2-GP I knockout mice are reported to have impaired thrombin generation (Sheng et al. 2001b), two human siblings, wholly deficient in β_2-GP I had normal markers for thrombin generation and fibrin turnover (Takeuchi et al. 2000). It is known that β_2-GP I is expressed on normal placentas, (Donohoe et al. 2000) and that its placental expression is reduced in APS. However, the knockout mouse

model has shown that, β_2-GP I is not essential for successful pregnancy in mice, although it is required for optimal placental development and fetal growth (Robertson et al. 2004). It therefore seemed unlikely that an antibody induced deficiency of β_2-GP I was responsible for the pathogenic effect of the antibody. Indeed, the levels of β_2-GP I antigen are actually increased in APS (McNally et al 1995a).

Galli et al (1995) suggested that prothrombin and β_2GPI dependent antibodies could be differentiated by means of the KCT and dRVVT tests. However, this was based on the ability of cardiolipin vesicles to adsorb aPA, rather than a genuine demonstration of anti prothrombin and or anti-β_2GPI activity.

Binding of anti- β_2GPI to β_2GPI is intrinsically of low affinity and the binding is dependent on the density of the antigen rather than neo-epitope formation (Sheng et al 1998). Anti-β_2GPI has been shown to enhance the binding of β_2GPI to phospholipids (Takeya et al. 1997). It was thought that this was due to the ability of anti- β_2GPI to form divalent complexes of β_2GPI on the phospholipid surface (Sheng et al 1998; Willems et al. 1996). Due to the increased affinity, β_2GPI is then able to compete with clotting factors for the phospholipid surface, resulting in the in vitro prolongation of coagulation (Takeya et al 1997). This was substantiated by the finding that covalently bound dimers of β_2GPI mimic the in vitro effects of β_2GPI anti- β_2GPI antibody complexes (Lutters et al. 2001).

1.5.5 anti-prothrombin

It was initially thought that anti-prothrombin antibodies were not associated with venous thromboembolism (VTE) or recurrent pregnancy loss (Forastiero et al 1997) (Donohoe et al 2001). However, complexes of anti-prothrombin antibodies and prothrombin cause lupus anticoagulant activity by competing with the binding of

clotting factors for catalytic phospholipid surfaces (Simmelink et al. 2001) in a mechanism similar to that of anti- β_2GPI. Anti-prothrombin antibodies appear to be heterogeneous (Atsumi et al. 2000) (Horbach et al. 1998a) and their detection is highly dependent upon the assay system used (Donohoe et al 2001). At present, the standardisation of anti-prothrombin antibody assays is poorly standardised and this may be hindering progress in this area (Galli et al 2003a).

1.5.6 inhibition of activated protein C

It has been proposed that aPA dependent inhibition of the protein C pathway may have a role in the pathogenesis of antiphospholipid syndrome and several mechanisms have been suggested:

- Some aPA may directly inhibit protein C and/or protein S (Malia *et al 1990*).
- β_2 GPI is known to bind to protein C and modulate its activation (Keeling *et al* 1993).
- It has been claimed that aPA may inhibit the thrombomodulin-dependent activation of protein C (Cariou et al. 1988; Keeling et al. 1993; Oosting et al 1993b) but this has been disputed by others (Potzsch et al. 1995)
- It has been proposed that some β_2 GPI dependent antibodies bind to protein C/APC possibly stabilising a β_2 GPI- APC complex preventing its action (Atsumi et al. 1998).
- Anti-β_2 GPI but not anti-prothrombin antibodies has been reported to inhibit the inactivation of factor V by activated protein C (Galli *et al* 1998).

Galli *et al* (1998) suggested that only β2GPI dependent aPA, and not anti-prothrombin antibodies, were responsible for the inhibition of APC, and this view has been supported by others were (Martinuzzo et al. 1996; Mercier et al. 1998; Viveros et al. 2005).

Another group has reported that 'anticardiolipin' antibodies from patients with APS may cross-react with prothrombin and thrombin (Hwang et al. 2001), APC (Hwang et al. 2003), factor Xa (Yang et al. 2006) and plasmin (Yang et al 2004) and have suggested that these antibodies prevent APC, antithrombin and plasmin from binding to their substrates through direct binding to the inhibitor or its substrate. However, the work hinges on the results obtained using seven monoclonal antibodies, which show remarkable cross-reactivity with many coagulation molecules.

It was against this background of poor standardisation of assays for aPA and the lack of agreement over their pathological mechanisms, that I hypothesised that the measurement of the haemostatic phenotype rather than the traditional immunological markers may be more informative in these patients.

1.6 Antiphospholipid antibody interference in laboratory screening tests

As the definition of a lupus anticoagulants (LA) is an antibody that interferes with phospholipid dependent coagulation reactions in vitro (Brandt et al 1995), it is not surprising that aPA are associated with falsely abnormal results in other diagnostic tests. Interference in the APTT was first described by Okpara et al (1977) who reported that plasmas from patients with lupus anticoagulants prolonged both the APTT and the tissue thromboplastin inhibition test, and that this was not corrected by mixing with normal plasma. They also showed that the maximum rate of change of optical density during clotting was reduced when compared to normal plasma. APTT reagents differ widely in sensitivity to LA (Jennings et al. 2002), and this is largely dependent on the lipid composition, both in terms of the amount and the type of phospholipid present.

While we have been aware of the sensitivity of the dilute thromboplastin test to lupus anticoagulant for many years (Schleider et al 1976), the susceptibility of the

prothrombin time, and hence the International Normalised Ratio, to LA is more controversial. Moll et al (1997) showed interference with some reagents, while Lawrie et al (1997) reported that aPA had no effect. It is now generally accepted that antibodies from some patients with LA can cause a false elevation in INR with certain reagents (Della et al. 1999; Robert et al. 1998). Tripodi et al, (2001) recommended that new thromboplastins, especially those made of relipidated tissue factor, should be checked for their responsiveness to LA before they are used to monitor oral anticoagulant treatment in patients with APS.

1.7 Aims of this thesis

The central hypothesis of this thesis was that acquired resistance to APC could be important in the pathogenesis of APS and that the global assessment of the coagulation and protein C anticoagulant pathways might be more informative than the traditional immunological markers.

The aims of this study were to:

- Investigate the frequency of protein C pathway defects in patients with aPA and to study clinical correlates.
- Investigate the mechanisms of antiphospholipid interference in the protein C pathway, with particular respect to phospholipid composition and β_2-GPI dependency.
- Assess the APC resistance in patients with aPA in terms of thrombin generation.

Chapter 2 Methods

2.1 Blood collection

Blood collection and plasma preparation were performed in the same manner throughout this study. Venous blood was collected into 5 ml tubes containing a one-tenth volume 0.105 M tri-sodium citrate (e.g. Vacutainer®, Becton Dickinson, RPlymouth, UK) using 19 or 21 gauge needles and minimal stasis. Platelet poor plasma was prepared by double centrifugation at room temperature at 2000g for 15 minutes, and frozen in aliquots at -80°C until assayed. A buffy coat sample was also collected for PC confirmation of factor V Leiden status. Ethical consent was obtained and all patients and controls gave informed consent.

2.2 Protein C pathway screening tests

The following four screening tests were used:

a) ACTICLOT® V-OUT (American Diagnostica, Greenwich, CT, USA)

b) PCA test (Diagnostic Reagents Ltd, Thame, UK)

c) GradiThrom PCP (Gradipore, North Ryde, Australia)

d) ProC® Global (Dade Behring GmbH, Marburg, Germany)

a) V-OUT uses two snake venoms, the first of which activates endogenous protein C while the second initiates clotting. The manufacturer declined to identify the snake venoms used, but as they stated that one activated protein C, and the other activated factor X, it was most likely that the venoms were an extract from *Agkistrodon contortrix contortrix* (Southern Copperhead, also known as Protac® or protein C activator) and *Vipera russelii* (Russell's viper venom). Unlike the other three tests, exogenous APC is also utilized in this test. Briefly, equal volumes of plasma and the APC reagent (containing both protein C activator and exogenous APC) or distilled water were

incubated at 37°C for 2 minutes prior to the addition of the clotting reagent. A ratio of clotting time for patient plasma with APC reagent/clotting time of patient plasma with water was calculated.

b) In the PCA test, a ratio of clotting times obtained using an activated partial thromboplastin time (aPTT) reagent and the same aPTT reagent containing Protac® is calculated. Briefly, equal volumes of plasma and aPTT or aPTT/PCA are incubated at 37°C for 5 min prior to the initiation of clotting by $CaCl_2$ diluted in saline. Predilution of plasma 1:5 in factor V-deficient plasma (Diagnostic Reagents) was performed for confirmation of factor V Leiden.

c) The PCP test uses Protac® to prolong clotting initiated by a phospholipid-rich dilute Russell's viper venom reagent containing $CaCl_2$ (PRVV). The test is simultaneously performed with the substitution of saline for Protac®, and a ratio of the two measurements is reported. Equal volumes of plasma and Protac® or saline were incubated at 37°C for 5 minutes prior to the initiation of clotting by PRVV. Ratios of ≤ 1.5 are usually due to factor V Leiden, and ratios between 1.5 and 2.5 are suggestive of protein C or protein S deficiency.

d) ProC Global also measures the degree of prolongation of the aPTT caused by the activation of endogenous APC by a protein C activator (venom from Agkistrodon contortix). The CA-6000 coagulometer (Sysmex) incubates equal volumes of plasma with aPTT reagent and buffer or protein C activator at 37°C for 3 min. Clotting is then initiated by the addition of $CaCl_2$. Clotting times with and without protein C activator were measured in parallel. Standard Human Plasma (Dade Behring) was used to calculate a Normalised Ratio (NR) using a lot specific calibration factor.

$$\text{Normalised Ratio (NR)} = \frac{\left(\frac{\text{PCAT}}{\text{PCAT/0}}\right)_{\text{sample}}}{\left(\frac{\text{PCAT}}{\text{PCAT/0}}\right)_{\text{SHP}}} \times \quad \text{sensitivity of SHP}^{*}$$

Where PCAT = clotting time with activator, PCAT/0 = clotting time with buffer, SHP = standard human plasma, and sensitivity = calibration factor for standard human plasma. For factor V Leiden screening, a 1:5 predilution of plasma in factor V-deficient plasma (Dade Behring) was performed to minimize the influence of factors other than factor V Leiden on the clotting times. NR < 0.8 was given as the decision limit for detecting factor V Leiden or protein C and protein S deficiencies.

Quality control was achieved by analysing the following lyophilised plasma samples:; locally prepared pooled normal plasma (double centrifuged and prepared from at least 20 healthy normal subjects); a frozen pool of plasmas from donors confirmed as heterozygous factor V Leiden (Precision Biologicals, Dartmouth, Nova Scotia, Canada).

2.3 Protein C assay

Protein C was assayed by an amidolytic method using *Protac*® and protein C substrate (Unitrate™ PC, Technoclone) and calibrated against a standard plasma (Technoclone).

2.4 Protein S assay

Free protein S was assayed by ELISA using a capture monoclonal antibody specific for free protein S and a polyclonal peroxidase conjugated detection antibody (Corgenix, Westminster, Co, USA) and calibrated against the international standard plasma 93/590 (NIBSC, UK).

2.5 Factor V Leiden mutation analysis

DNA extracted from whole blood buffy coats was analysed for the factor V Leiden mutation by polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (Bertina et al. 1994). A 267-basepair segment of the factor V

gene was amplified using specific primers and the PCR products were digested with a specific restriction enzyme Mnl1, separated electrophoretically and visualised using ethidium bromide.

2.6 Activated protein C resistance test (Commercial clotting test)

APCR was determined using the Coatest® Activated Protein C Resistance method with predilution in FV deficient plasma (Chromogenix, Mölndal, Sweden). Briefly, APTT was measured using an ACL 3000 coagulometer (Instrumentation Laboratory) in the presence and absence of APC and the results were expressed as a ratio of the two clotting times. Unless otherwise stated, APCR was performed without prediluting the patient plasma in factor V deficient plasma, i.e., the unmodified APCR test.

2.7 Antiphospholipid antibody detection

Lupus anticoagulant activity was assessed by the dilute Russell's viper venom time dRVVT method (Thiagarajan et al 1986) using the UNITEST LA – dRVVT/PNR kit (Technoclone). In accordance with the BCSH guidelines (Greaves et al 2000), mixing studies using pooled normal plasma, and a platelet neutralisation procedure were performed on all samples giving a dRVVT ratio above the local normal reference range. A prolongation of the 50:50 mix, and a correction of the ratio by >10% or to within the normal range was considered positive for LA (Gardiner et al. 2000)

Anti-cardiolipin antibodies were measured by ELISA using a method based on that of (Loizou et al 1985). Microtitre plates were coated with cardiolipin, and non-specific binding was eliminated by blocking the wells with 10% adult bovine serum. Sera diluted 1:50 were incubated in the wells and, after washing to remove unbound immunoglobulin, IgG and IgM were detected using enzyme conjugated antisera.

Both IgG and IgM anti-β_2-glycoprotein I antibodies were assayed using a commercially available ELISA (DIASTAT™, Euro-Diagnostica, Arnhem, Netherlands) according to

the method of McNally *et al* (1995b). Briefly, diluted serum or plasma are added to microtitre plates coated with purified human β_2-GPI. Bound immunoglobulin is detected by means of an enzyme labelled monoclonal antibody specific for IgG or IgM.

2.8 Endogenous APC ratio (EAPCR)

Acquired resistance to APC was assessed by the degree of prolongation of the clotting time, produced by activated endogenous protein C in a modified dRVVT with dilute phospholipid. RVV and 0.2 U/ml Protac® (Technoclone). Two phospholipid preparations were used: Bell and Alton platelet substitute (Diagnostic Reagents Ltd), a phospholipid source known to be rich in the zwitterionic phospholipids phosphatidylethanolamine and sphingomyelin; and Actin FS (Dade Behring), which is 'lupus insensitive'(Brancaccio et al. 1997; Lawrie et al. 1998). The Bell and Alton was diluted 1/2 and Actin FS was diluted in imidazole buffer prior to use. At these dilutions, the final total phospholipid concentrations were approximately the same. The phospholipid content of a single lot of both reagents is shown inTable1. These were not the reagent lots used in this thesis, so I have assumed that there is reasonable consistency between batches.

Table 1: Phospholipid content of preparations used μg/ml.
From Kitchen et al (1999)

Testing was performed on the CA-6000 coagulometer (Sysmex), which uses light scatter at 660 nm to detect clot formation. The phospholipid was titrated to give the optimal prolongation of clotting time by activated protein C, yet retain sensitivity to antiphospholipid antibodies. Two aliquots of plasma were pre-incubated with dilute phospholipid, prior to the addition of Protac to one aliquot, and imidazole buffer (0.05M, pH 7.30) to the other. Following a 5 minute incubation, dilute RVV containing 0.025M $CaCl_2$ was added and the clotting times were recorded. The method is shown in figure 8. Results were expressed as a normalised ratio (EAPCR) against pooled normal plasma (PNP).

$$\text{EAPCR} = \frac{\text{test with Protac}^{®}/\text{PNP with Protac}^{®}}{\text{test with buffer/PNP with buffer}}$$

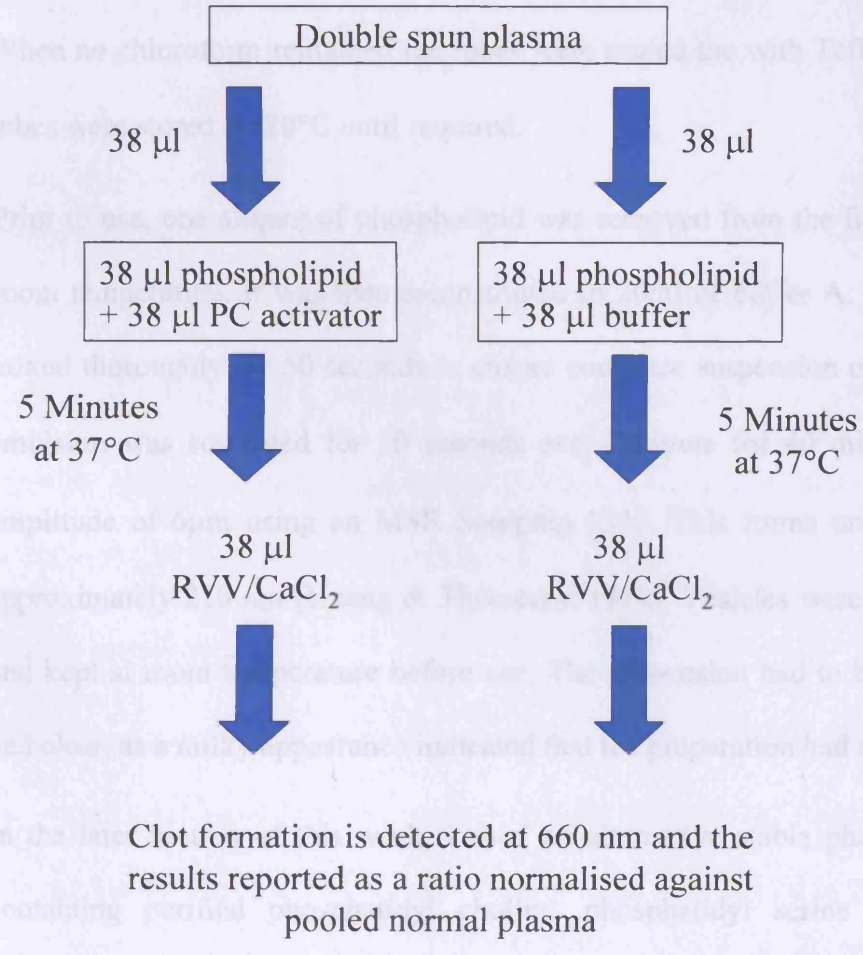

Figure 8 Endogenous APC resistance method

2.8 Phospholipid vesicle preparation

Di-oleyl-phosphatidylethanolamine (DOPE), di-oleyl-phosphatidylserine (DOPS) and di-oleyl-phosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Birmingham AL, USA), dissolved in chloroform.

The following chloroform dissolved lipids were dispensed into a clean dry 25 ml volumetric flask;

5.0 ml of 10mg/ml DOPE

5.0 ml of 10mg/ml DOPS

15.0 ml of 10mg/ml) DOPC

After thorough mixing, 300 µl aliquots were pipetted into Pyrex™ culture tubes (Corning). In a fume cupboard, the phospholipids were dried under flowing nitrogen. When no chloroform remained the tubes were sealed the with Teflon™ screw tops. The tubes were stored at -80°C until required.

Prior to use, one aliquot of phospholipid was removed from the freezer and brought to room temperature. It was then reconstituted in 20ml of buffer A. The tube was vortex mixed thoroughly for 60 seconds to ensure complete suspension of phospholipids. The emulsion was sonicated for 30 seconds every minute for 40 minutes at 27°C at an amplitude of 6µm using an MSE Soniprep 1500. This forms unilamellar vesicles of approximately 210 nm (Huang & Thompson 1974). Vesicles were prepared fresh daily and kept at room temperature before use. The suspension had to be entirely colourless and clear, as a milky appearance indicated that the preparation had deteriorated

In the later section of this work, I used an alternative stable phospholipid emulsion, containing purified phosphatidyl choline, phosphatidyl serine and sphingomyelin (kindly provided by Steffen Rosen, Rossix, Mölndal, Sweden). Due to the relatively

noisy signal produced, this was not suitable for studying the kinetics of thrombin formation, but was perfectly acceptable for measuring the total thrombin generation.

2.9 Sub-sampling thrombin generation method

Reagents

The following reagents were used:

Buffer A, 20 mM Tris buffered saline containing 0.5% bovine serum albumen pH 7.35,

Buffer B, 20 mM EDTA in buffer A pH 7.9

Buffer C, 20mM Tris buffered saline pH 7.35

Thrombin substrate (Unitrate THR 2AcOH.H-D-CHG-Gly-Arg-pNa, Technoclone) was reconstituted in 1 ml of distilled water and further diluted in buffer A to give a 5 mM concentration.

Ancrod (Sigma Aldrich, product number A5042) was reconstituted in distilled water and diluted to 5U/ml and frozen in 0.5 ml aliquots at -80°C.

Activated protein C (Eli Lilly, lot: RS0289). Frozen at 500 nM at -80°C. Working strength of 108 nM was achieved by diluting 1/5 in buffer A.

Calcium Chloride (VWR) was diluted to 100 mM in buffer A (1/10).

Innovin; relipidated human recombinant tissue factor (Dade Behring) was diluted 1/32 in 100mM $CaCl_2$ immediately prior to use.

Plasma preparation

Plasma was treated using Ancrod, an enzyme isolated from the venom of the Malaysian pit viper (Agkistrodon rhodostoma), which causes defibrination by the limited proteolysis of fibrinogen. 20 µl of 5 U/ml Ancrod were added to 600 µl of plasma in a 1.5 ml microcentrifuge tube. The mixture was mixed with a wooden applicator and incubated in a 37° water batch for 10 minutes, then placed on ice for a further 10

minutes. The fibrin clot was removed by winding onto the applicator and excess plasma was squeezed onto the side of the tube. The defibrinated plasma was microcentrifuged at 10,000 ***g*** for 3 minutes and placed on ice until assayed.

Measurement

The reagents were used at the following concentrations

Final reaction mixture	**Working concentrations**
Final Concentration	Working concentration
10 nM APC	500nM stock diluted 200µl in 1 ml of buffer A
1/192 Tissue factor	Innovin diluted to 1/32 in 100 mM $CaCl_2$
16mM $CaCl_2$	100 mM
25 µM phospholipid vesicles	400 µM DOPE, DOPS, DOPC 20:20:60

240 µl of defibrinated platelet poor plasma and 20 µl of the phospholipid vesicle suspension were pipetted into a siliconised glass cuvette with constant stirring at 37°C (1000 rpm using the PAP4 platelet aggregometer, Biodata Corp, Philadelphia, USA). After 2 minutes incubation, 10 µl of the reaction mixture was pipetted into 300 µl of buffer B and mixed by gentle vortex mixing. The presence of EDTA prevented any further reaction. This was the time zero sample. The reaction was started by the addition of 60 µl of the calcium/tissue factor mixture. 10 µl of this reaction mixture was subsampled into 300 buffer B as described above, at the following times intervals: 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 and 600 seconds

The subsampled aliquots in buffer B were transferred into a micro-cuvette and processed using an ACL 300 in 'Research mode'. Thrombin activity was assessed by measuring the change in optical density at 405 nm, due to cleavage of 5mM thrombin substrate, using the following instrument protocol:

100 µl sample, 25 µl buffer B and 25 µl substrate, inter-ramp 1 s, time delay 0 s and acquisition time 120 s. The data were transferred to a computer and the changes in optical density over time were calculated (using the Chromo.dat path in the research software).

Two methods of assessing thrombin generation were used:

Thrombin activity was plotted against time and the lag phase prior to the start of thrombin formation, the time of peak thrombin formation and the relative size of the thrombin peak were calculated.

As the endogenous thrombin potential of a given sample is known to be directly proportional to the residual amidolytic activity of α_2 macroglobulin-bound thrombin (α_2M-IIa) (Nicolaes et al 1997) (Tans et al. 2000), the degree of APCR was measured by comparing the α_2M-IIa levels in the presence and absence of rhAPC. Resistance to APC was expressed as a normalised APC resistance ratio (nAPCsr) which was calculated as follows:

$$nAPCsr = (\alpha_2M\text{-}IIa_{+APC}/\alpha_2M\text{-}IIa_{-APC})_{sample} / (\alpha_2M\text{-}IIa_{+APC}/\alpha_2M\text{-}IIa_{-APC})_{normal\ plasma}$$

2.10 Automated thrombin generation method

Reagents

The following reagents were used for this method:

Innovin; lyophilised recombinant human tissue factor (Dade Behring)

Pefachrom® TG, a slow acting thrombin specific substrate; H-β-Ala-Gly-Arg-pNA (Pentapharm, Basel, Switzerland)

Human recombinant activated protein C (rhAPC) (Eli Lilly, Indianapolis, IN, USA);

The phospholipids (DOPS:DOPE:DOPC) were prepared as for the subsampling method

An alternative stable phospholipid emulsion, containing purified phosphatidyl choline, phosphatidyl serine and sphingomyelin (Rossix, Mölndal, Sweden) was also used.

Automated thrombin generation method

Three reagents are prepared for this method:

- Innovin used at a final dilution of 1/600 (approximately 7pM, as measured using the IMUBIND® Tissue Factor ELISA [American Diagnostica Inc, Stamford, CT] was mixed with $CaCl_2$ (final concentration 15mM) and phospholipid (final concentration 20 μM).
- Innovin, $CaCl_2$, phospholipid and rhAPC (final concentration 5nM)
- Pefachrome TG diluted to 3.3 nM

Thrombin generation was measured with and without exogenous rhAPC, using the ACL9000 (Instrumentation Laboratory, Milan, Italy). This is an automated centrifugal analyser, which can measure changes in optical density (OD) at 405 nm. Plasma was defibrinated by the addition of 0.2 U/ml Ancrod at 37°C for 10 minutes, followed by a further 10 minutes on ice and manual removal of fibrin, prior to analysis. Two start reagents were prepared, containing dilute tissue factor, $CaCl_2$ (final concentration 16 μM) and phospholipid (final concentration 20μM). rhAPC in tris buffered saline, pH 7.35 (final concentration 5nM) was added to one start reagent, while only buffer was added to the other. After warming the plasma, start reagent and thrombin substrate to 37°C, the coagulometer mixed the reactants together and measured the optical density at 405nm at 2-second intervals over a period of 1000 seconds. The short time interval between measurements results in a 'noisy' signal, which was overcome by using a moving average algorithm to smooth the signal. The raw data was transferred to a personal computer, imported into Microsoft® Excel®, and, following curve smoothing, using a simple moving average (the unweighted mean of the last 8 data points), the ETP

was calculated from the area under the curve as described by Hemker and Beguin (Hemker & Beguin 1999). This allowed the data to be displayed cumulatively or as the rate of thrombin generation against time, and the calculation of total thrombin generation, lag phase to the start of thrombin generation and peak thrombin generation. This provided important information about the kinetics of thrombin formation. Total thrombin formed, with and without 5nM rhAPC, were expressed as ratios relative to pooled normal plasma (PNP) as suggested by Rosing et al (2004)

ETP = thrombin formed in patient plasma/thrombin formed in PNP, with no APC

ETP^{+APC} = thrombin formed in patient plasma /thrombin formed in PNP, with 5nM APC

2.11 Prothrombin assay

Plasma prothrombin levels were measured in a one-stage clotting assay using factor II immunodepleted plasma (Technoclone) and Thromborel S (Dade-Behring, Germany).

2.12 Factor V Leiden and prothrombin gene mutation analysis

Factor V Leiden and prothrombin gene mutation (G20210A) were assessed by PCR and electrophoresis (Poort et al 1996, Bertina 1997).

2.13 TFPI antigen ELISA

Total TFPI antigen was assayed using the IMUBIND® Total Tissue Factor Pathway Inhibitor ELISA kit (American Diagnostica). This is a quantitative sandwich ELISA employing a rabbit anti-human TFPI polyclonal capture antibody and a biotinylated monoclonal antibody, specific for the Kunitz domain 1 of TFPI. This assay recognises native, truncated and conjugated forms of TFPI

2.14 Automated TFPI sensitivity index

In order to assess TFPI function, I adapted a method from (Dahm et al. 2005). The method was in essence a dilute thromboplastin time, in which the plasmas were

incubated in the absence and the presence of saturating amounts of a rabbit polyclonal antibody, that blocked TFPI function (American Diagnostica Inc). It was found that a concentration of 250 mg/ml and an incubation time of 15 minutes gave optimal inhibition of TFPI function (Table 2). This is much longer than the original method with this antibody, perhaps reflecting differences in the avidity of the antibodies used. Testing was performed on the ACL7000 by incubating the treated plasma with human recombinant tissue factor (Innovin, Dade Behring) diluted at 1:300 in tris buffered saline for 5 minutes. Coagulation was triggered by the addition 35mM CaCl2. Results were expressed as a ratio with the clotting time in the presence of anti-TFPI as the denominator. The ratio was normalized against a ratio obtained with pooled normal plasma. Due to the exceptionally low phospholipid concentration used, this test is exquisitely sensitive to residual platelet contamination, so double centrifugation of plasma was essential. Acceptable within run and between run imprecision was obtained for the dilute thromboplastin clotting times with and without anti-TFPI antibody; percentage coefficient of variation 1.86% and 1.39% (within run); 2.20% and 2.48% (between run) respectively.

Table 2: Titration of anti-TFPI antibody

Ab dilution	Stock 250 mg/ml	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	Plasma only
Run 1	64.2	70.7	72.4	69.9	72.2	71.7	71.4	72.7	71.7	74.4
Run 2	65.4	66.2	67.4	68.9	70.2	70.7	70.9	71.4	71.4	75.2
Mean	64.8	68.45	69.9	69.4	71.2	71.2	71.15	72.05	71.55	74.8

2.15 Anti-TFPI antibody detection by ELISA

Anti-TFPI antibodies were measured by ELISA using the following method based on that of Wolberg et al (2004) . Polystyrene microtitre plates (Nunc Maxisorp, Nalge Europe Ltd. Hereford, UK) were coated with full-length recombinant TFPI (Chiron Corporation, Emeryville, CA, USA) at 5 mg/mL in 0.1M carbonate/bicarbonate buffer,

overnight at 4°C. The wells were washed in phosphate buffered saline (PBS - pH 9.6) containing 0.05% Tween-20. The wells were blocked with PBS/tween buffer containing 1% bovine serum albumin for 1 hour at room temperature. Samples, diluted 1:100 in the blocking buffer, were added to the wells and incubated at room temperature for 2 hours. After washing in PBS/Tween, bound IgG or IgM were detected by the addition of alkaline phosphatase-conjugated goat anti-human IgG or IgM (Sigma Aldrich, Poole, UK), diluted 1:2000 in blocking buffer. After 2 hours incubation, the plates were washed and 1 mg/mL disodium hexahydrate phosphatase (Sigma) diluted in 1M diethanolamine buffer (pH 9.8, Sigma) was added to the wells. After 20 minutes, the reaction was stopped by the addition of 3M sodium hydroxide and absorbance was measured at 405nm. Normality was defined as the 99th percentile of 25 normal healthy subjects.

2.16 Factor VIII assay

Plasma factor VIII concentration was measured by a one-stage APTT based clotting assay, using Pathromtin SL (Dade Behring) and immunodepleted factor VIII deficient plasma (Technoclone). All assays were performed at three dilutions to ensure linearity using the multi dilution analysis function of the CA-1500 coagulometer (Sysmex).

2.17 Affinity *purification of immunoglobulin IgG*

IgG fractions from patients with APS and from normal healthy individuals were purified using a protein G sepharose column (Amersham Biosciences, Little Chalfont, UK). Briefly, following equilibration with PBS, defibrinated plasma or serum was applied to the column, which was then washed with PBS. Immunoglobulin was eluted using 0.1 M glycine at pH 2.8, and immunoglobulin fractions were detected by measuring absorbance at 280nm. IgG was quantitated assuming an extinction coefficient of 13.6 at 280nm.The fractions were dialysed using an Amicon Mini-

Ultrafiltration Cell across a PM30 membrane into PBS, or desalted using Sephadex G25 gel filtration (Amersham Biosciences) and freeze-dried in 16 mg/ml mannitol. The freeze-dried preparations were dissolved directly into defibrinated PNP for the IgG spiking experiments.

2.18 Affinity *purification of immunoglobulin IgM*

Purification of IgM can be problematical as there is no single step procedure available, which does not result in low yield or a high degree of impurity. Initial experiments were performed using the method Roodbari et al (2003). Briefly, an IgM-rich fraction was prepared by precipitating with polyethylene glycol 6000 (PEG-6000). This employed a two-step procedure to eliminate unwanted proteins and prepare an IgM-rich fraction. First, serum or defibrinated plasma was diluted 2.5-fold in 20 mM potassium phosphate buffer, pH 7.4, and was placed in a magnetic stirrer. Then 40% PEG solution was added dropwise to achieve a final concentration of 3% PEG in the mixture. After 1/2 h of gentle stirring, the mixture was centrifuged at 3,000***g*** for 15 min and the supernatant was collected. In the second step, a 40% PEG solution was added as in step 1 to reach a final concentration of 8%. The solution was incubated overnight in a refrigerator, and IgM-rich precipitants were separated by centrifugation and kept at 4°C. This was then purified using a sephacryl column (see next section). However, preliminary experiments produced a poor yield and this approach was abandoned.

Sephacryl Column separation

A Sephacryl™ S-300 HR was prepared by suspending in 20:80 ethanol/PBS and allowing the column to slowly settle in order to eliminate air bubbles. The column was then equilibrated with 1% Sodium azide/PBS. 10 ml of plasma was defibrinated with Ancrod, introduced to the column and eluted at a rate of 1.5ml/minute. 7 ml fractions were collected and protein content was assessed by measuring OD at 280nm using an ultraviolet spectrophotometer. The relative IgM content was measured using an IgM

enzyme linked immunosorbent assay (ELISA). Further purification was achieved using a thiophilic adsorption medium (2-mercaptopyridine coupled to Sepharose, Amersham Hi-Trap IgM column, GE Healthcare Life Sciences, Amersham, UK). Desalting was achieved using a Sepaphdex™ G-25 column (PD-10 desalting column GE Healthcare Life Sciences) equilibrated in 16mM mannitol. The purified IgM fractions were eluted in 16nM mannitol by gravity. Protein concentration was quantitated assuming an extinction coefficient of 14 at 280nm. 5ml aliquots of purified IgM were freeze-dried in 16 mg/ml mannitol. The freeze-dried preparations were dissolved directly into defibrinated PNP for the IgM spiking experiments.

Chapter 3. Factors influencing commercial protein C pathway screening tests

3.1 Introduction

Routine thrombophilia screening generally requires the assay of protein C, protein S and APC resistance/factor V Leiden. Accurate diagnosis may require the use of antigenic, chromogenic and clotting assays in order to exclude or confirm a defect. These assays are frequently technically difficult, time consuming and expensive. At the inception of my thesis, several manufacturers had developed methods for the global assessment of the protein C pathway. These tests therefore appeared to provide a simple way of identifying aPA patients with defects in the protein C pathway for further investigation.

When first muted in the literature, the idea of a single global screening test for defects in the natural anticoagulant pathways seemed an appealing idea. However, the validity of the claims made regarding the sensitivity and specificity of these assays, had not been independently verified and the issue of aPA interference had not been addressed. As aPA are known to affect so many other phospholipid dependent coagulation tests, at the very least, one would expect the baseline clotting test to be prolonged by LA activity in some instances. It has been known for some time that functional protein S assays can give falsely low results compared to antigenic assays in the presence of LA (Lawrie et al. 1995). It was reported at the time that the results could not be explained by activated protein C resistance, and that lupus anticoagulant caused prolongation of the baseline clotting time within the protein S activity assay. However, Malia *et al* (1990) reported that some antiphospholipid antibodies inhibited the activated protein C/protein S complex in vitro.

In order to establish the best way to study aPA interference in the protein C pathway, I decided that it made sense to first study the tests already available for assessing the protein C pathway, and determine the factors affecting these assays.

3.2 Materials and methods

Samples for this study were collected from both UCLH and the Royal Hallamshire Hospital, Sheffield, in order to obtain sufficient samples from patients with congenital defects in protein C and S (which are relatively rare), who were not receiving oral anticoagulants.

3.2.1 Patients and samples

Reference ranges, from 40 healthy normal volunteers (pregnant women were excluded) for each method. In addition, reference ranges for the ProC Global (Dade Behring) and Diagen PCA (Diagnostic Reagents Ltd.) tests were determined with factor V deficient plasma correction. PT and APTT were also performed on all samples to exclude underlying coagulation abnormalities. As many of the clinical groups included patients receiving combined oral contraceptives (COC), it was thought inappropriate (at the time) to exclude women taking COCs from the control group. None of the patients were receiving heparin or warfarin, nor were they suffering from an acute thrombotic event at the time of testing.

Plasma samples from the following patients were tested

- 11 patients with congenital protein C deficiency
- 19 patients with congenital protein S deficiency
- 23 patients with the factor V Leiden mutation

- 20 patients investigated for thrombophilia as defined by the BCSH guidelines (Greaves et al 2001), but with no known anti-phospholipid antibodies or defects in the protein C system
- 16 samples from patients with LA as defined by the BCSH guidelines (Greaves et al 2000).

3.2.2 Global protein C pathway screening tests

The four screening tests studied were:

a) ACTICLOT® V-OUT (American Diagnostica)

b) PCA test (Diagnostic Reagents Ltd)

c) GradiThrom PCP (Gradipore)

d) ProC® Global (Dade Behring)

These were performed as described in the methods chapter

3.2.3 Statistical analysis

Instrument-specific reference ranges were used and, since most of the data groups in this study were skewed and this was not always corrected by log conversion, the 2.5th percentile was used to define normality. Non-parametric tests were employed throughout and the level of significance was taken as $P<0.05$.

3.3 Results

Acceptable within-run imprecision was obtained, with a coefficient of variation of <7% for all tests using a range of normal and abnormal controls. Between run imprecision was <10%. Reference ranges were established in forty healthy normal subjects. Plasma from two women believed to be using the oral contraceptive pill, and from another

subject with an acute phase reaction, gave values that were clear outliers (below the 2.5th percentile) by all methods and these results were subsequently excluded from the statistical analysis (table 3).

Table 3: Reference ranges from normal subjects (n = 37)

	V-OUT	PCA	PCP	ProC	ProC NR
Mean	5.56	13.51	7.47	3.34	1.05
SD	1.17	3.79	2.27	0.48	0.15
Mean –2SD	3.22	5.93	2.94	2.38	0.75
Median	5.27	13.16	7.30	3.31	1.04
5th percentile	4.20	7.01	4.31	2.70	0.85

NR =Normalised ratio

The characteristics of the patients with protein C deficiency, protein S deficiency, factor V Leiden and LA are presented inTable4. The dilute Russell's viper venom time (dRVVT) ratios reported were those obtained with the dilute phospholipid screening reagent. All patients selected because of a history of LA proved to positive again on this occasion. Protein S values are free protein S antigen levels determined by ELISA and protein C values are functional (chromogenic) levels. All factor V Leiden cases were detected using the Coatest APC resistance test, then were confirmed by RFLP and PCR.

Table 4: Characteristics of patients studied.

		Results		
Clinical group	Sex	Mean (standard deviation)	Range	Reference range
Congenital protein C deficiency [protein C (IU/ml)]	Male (*n* = 5)	0.60 (0.02)	0.57 – 0.63	0.70 – 1.30
	Female (*n* = 6)	0.56 (0.06)	0.46 – 0.62	
Congenital protein S deficiency [free protein S (IU/ml)]	Male (*n* = 5)	0.65 (0.05)	0.58 – 0.69	0.73 - 1.76
	Female (*n* = 14)	0.40 (0.15)	0.17 – 0.57	0.58 – 1.14
Lupus anticoagulant [dRVVT ratio]	Male (*n* = 6)	1.38 (0.25)	1.14 – 1.80	< 1.13
	Female (*n* = 10)	1.38 (0.22)	1.15 – 1.75	
Factor V Leiden [factor V Leiden phenotype]		Heterozygous	Homozygous	
	Male (*n* = 7)	5	2	
	Female (*n* = 16)	15	1	

a) No sex specificic differences in normal subjects were found with the V-OUT method so a single 2.5th percentile normal cut-off was used. Using this value, all factor V Leiden were correctly identified by the V-OUT method (figure 9). The V-OUT method was relatively insensitive to protein C deficiency [27% (3/11) identified. Only 35% (6/17) of protein S deficiencies gave results below the 2.5th percentile. 5% (1/20) of the thrombophilia samples and 30% (6/20) of the LAC samples gave abnormal results.

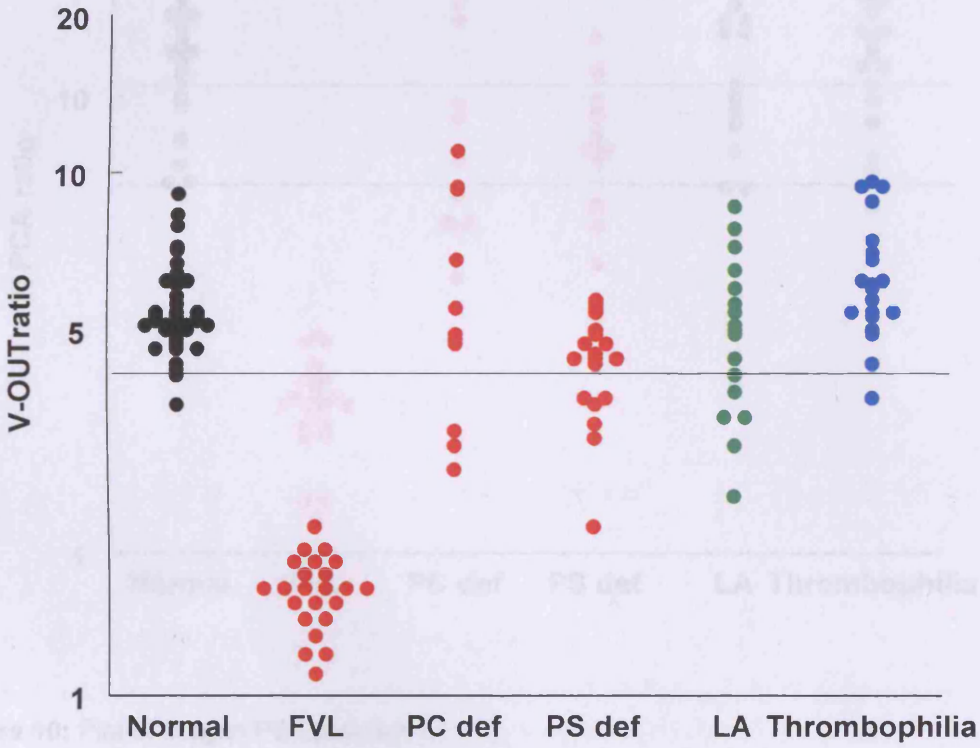

Figure 9: Plot of Acticlot V-OUT ratios.

Normal subjects, factor V Leiden (FVL), protein C deficiency (PC def), protein S deficiency (PS def), Lupus anticoagulant (LA) and thrombophilia. The broken line indicates the 2.5th percentile of normal.

b) All cases of factor V Leiden were correctly identified by the PCA method, with excellent discrimination between the normals and factor V Leiden samples observed (Figure 10). Abnormal PCA results were obtained in 55% (6/11) and 47% (8/17) of the protein C and protein S deficiencies, respectively, whereas unexpected abnormal results were obtained in 5% (1/20) and 35% (6/17) of the thrombophilia and LA positive samples, respectively.

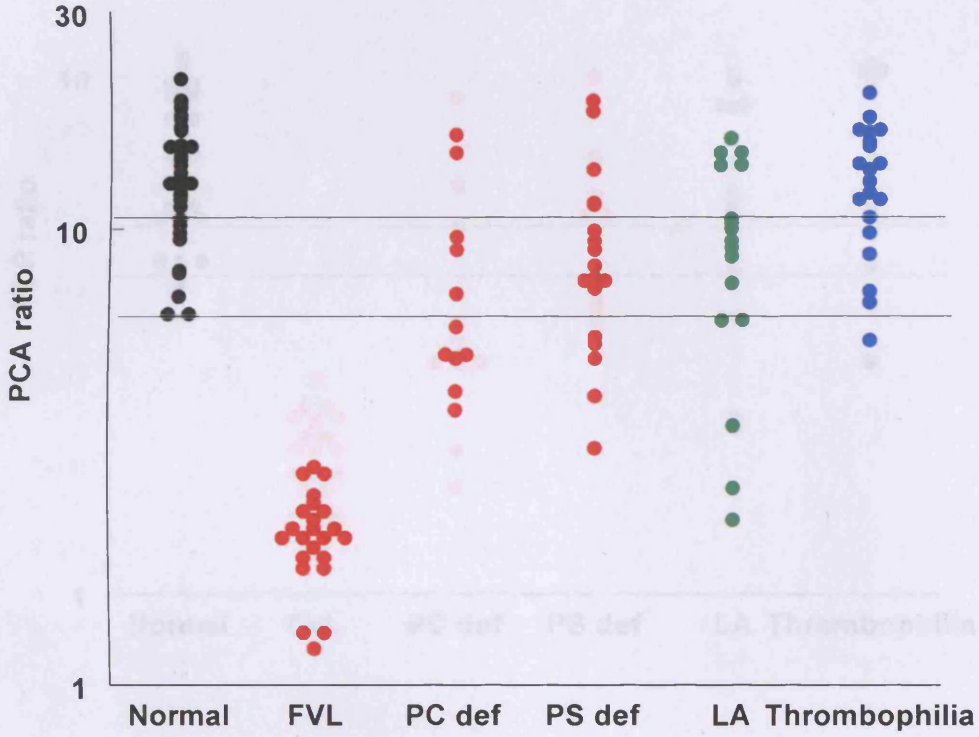

Figure 10: Plot of Diagen PCA test ratios.

Normal subjects, factor V Leiden (FVL), protein C deficiency (PC def), protein S deficiency (PS def), Lupus anticoagulant (LA) and thrombophilia. The upper broken line indicates the 2.5th percentile of normal males. The lower broken line indicates the 2.5th percentile of normal females.

c) All samples from patients with factor V Leiden fell below the 2.5th percentile of normal when tested with the PCP method (Figure 11). Seventy-three percent (8/11) of protein C deficiencies and 53% (9/17) of protein S deficiencies gave ratios below the 2.5th percentile. Ratios below the 2.5th percentile were obtained in 5% (1/20) of the thrombophilia samples and 29% (5/17) of the LAC-positive samples.

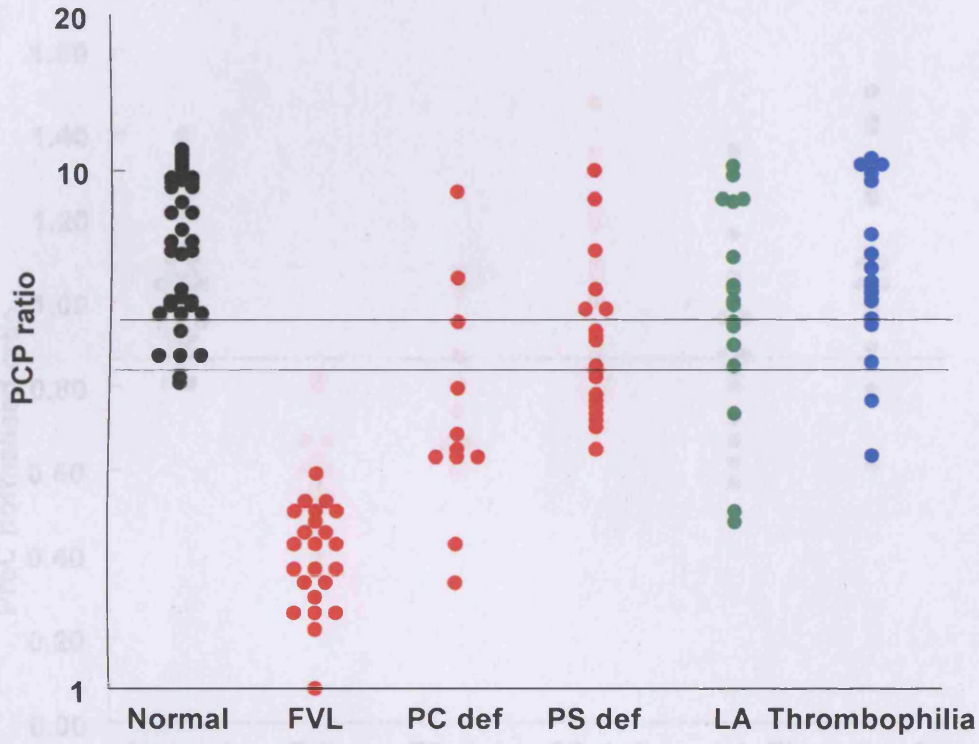

Figure 11: Plot of Gradipore PCP test ratios.

Normal subjects, factor V Leiden (FVL), protein C deficiency (PC def), protein S deficiency (PS def), Lupus anticoagulant (LAC) and thrombophilia (Thromb). The upper broken line indicates the 2.5th percentile of normal males. The lower broken line indicates the 2.5th percentile of normal females.

d) All ProC values were reported as normalised ratios (NRs). No sex specific differences were observed in the normal subjects, so a single 2.5th percentile value was used. All factor V Leiden samples were correctly identified (Figure 12). NR values less than the 2.5th percentile were observed in 55% (6/11) of protein C-deficient samples and 35% (6/17) of protein S-deficient samples. Low NR were observed in 10% (1/20) of thrombophilia sample and 35% (6/17) LA-positive samples.

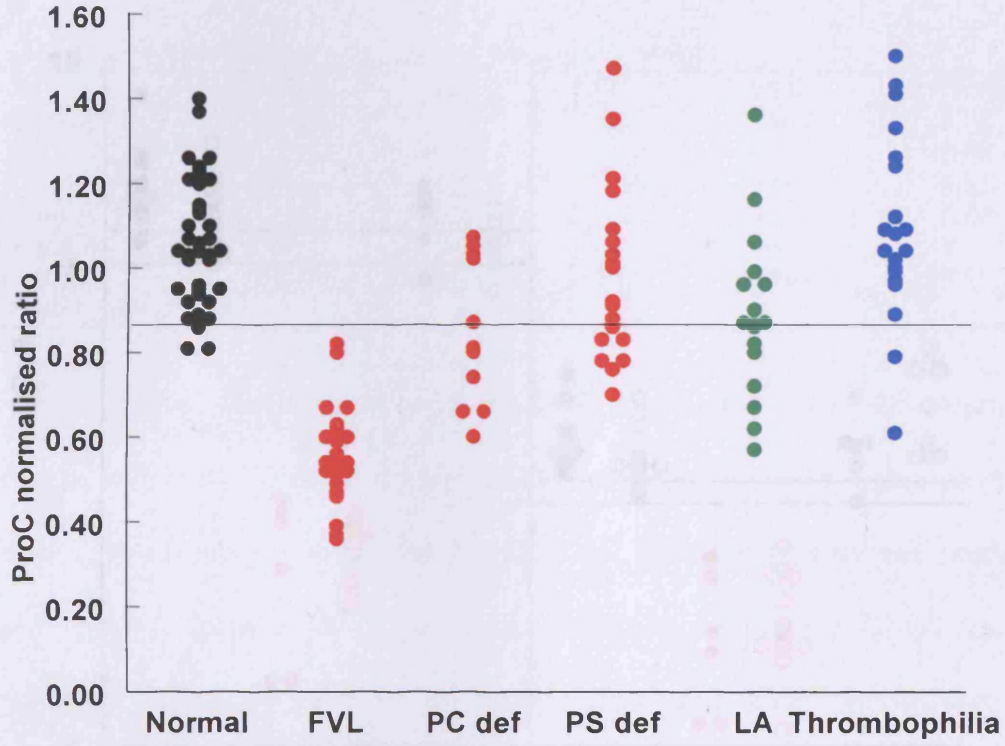

Figure 12: Plot of ProC Global normalised ratios.

Normal subjects, factor V Leiden (FVL), protein C deficiency (PC def), protein S deficiency (PS def), Lupus anticoagulant (LAC) and thrombophilia. The broken line indicates the 2.5th percentile of normal.

As expected, predilution with factor V-deficient plasma gave much tighter reference ranges when used with the PCA and ProC methods (Figure 13). All factor V Leiden plasmas were correctly identified by both methods, using the 2.5th percentile cut-off value. Furthermore, the three samples from homozygous factor V Leiden patients were clearly discriminated using predilution.

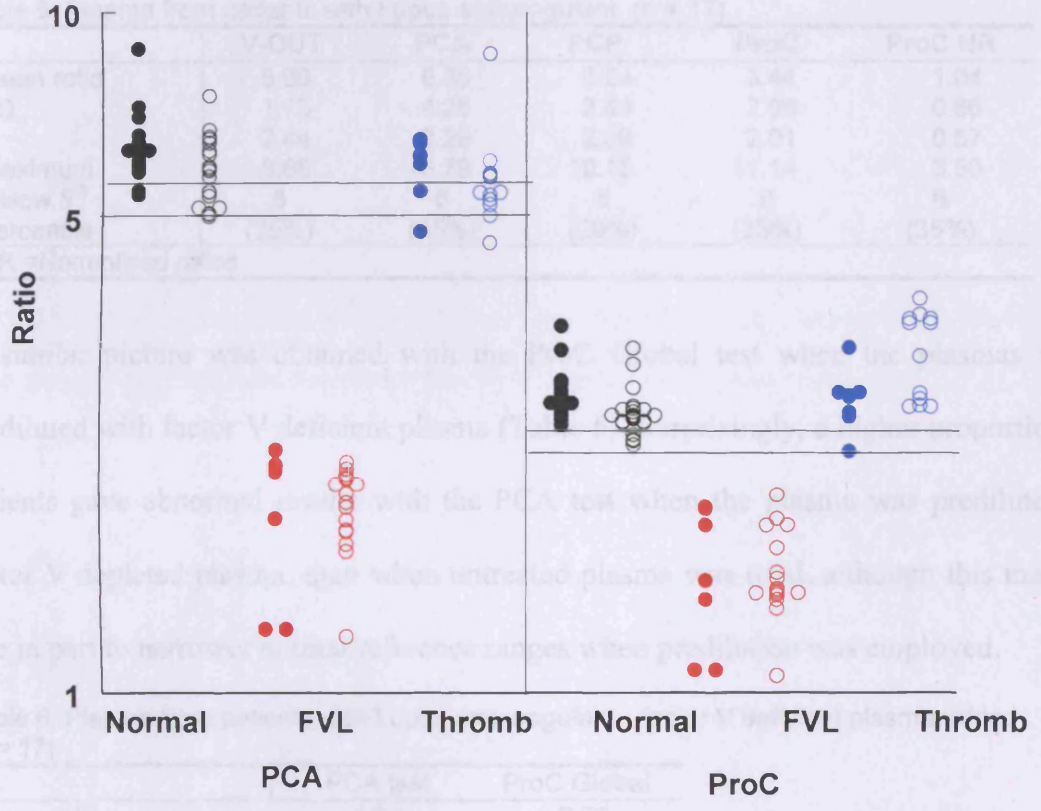

Figure 13: Diagen PCA test and ProC Global tests with predilution in factor V depleted plasma. Upper broken lines indicate the 2.5th percentiles for normal females and the lower broken lines show the 2.5th percentile for normal males. Open circles represent females and closed circle represent males.

Plasma samples from seventeen patients with lupus anticoagulant, but no evidence of specific defects in the protein C system, were studied. Ten patients had abnormal APTT results, and all had abnormal dRVVT. All four methods gave low ratios with 29 - 35% of samples (Table 5). There was considerable variation, between both the methods with different samples giving abnormal results with different methods. In many cases the abnormal ratio was due to prolongation of the baseline clotting time, i.e., the APTT or

venom clotting time without Protac® or exogenous APC. Not surprisingly, abnormal baseline clotting times for the PCA and ProC tests were found in those patients with abnormal APTT results (Table 6). However, some low ratios were caused by a reduced response to APC or Protac® resulting in only a mild prolongation in the clotting time by APC. Furthermore, in samples from three patients this finding was consistent with all four methods.

Table 5: Plasma from patients with Lupus anticoagulant (n = 17)

	V-OUT	PCA	PCP	ProC	ProC NR
Mean ratio	5.09	9.38	6.04	3.44	1.04
SD	1.73	4.25	2.49	2.08	0.66
	2.44	2.29	2.09	2.01	0.57
Maximum	8.65	15.79	10.15	11.14	3.50
Below 5th	6	6	5	6	6
percentile	(35%)	(35%)	(29%)	(35%)	(35%)

NR =Normalised ratios

A similar picture was obtained with the ProC Global test when the plasmas were prediluted with factor V deficient plasma (Table 6). Surprisingly, a higher proportion of patients gave abnormal results with the PCA test when the plasma was prediluted in factor V depleted plasma, than when untreated plasma was used, although this may be due in part to narrower normal reference ranges when predilution was employed.

Table 6: Plasma from patients with Lupus anticoagulant - factor V deficient plasma added (n = 17)

	PCA test	ProC Global
Mean ratio	4.84	2.76
SD	0.85	0.44
Minimum	2.92	2.18
Maximum	5.91	3.52
Below 5th percentile	10 (59%)	5 (29%)

In order to ascertain whether these abnormal ratios were as a result of LA activity or the inhibition of the APC pathway, I studied the individual clotting times and compared them to the reference ranges (Figures 14 and 15).

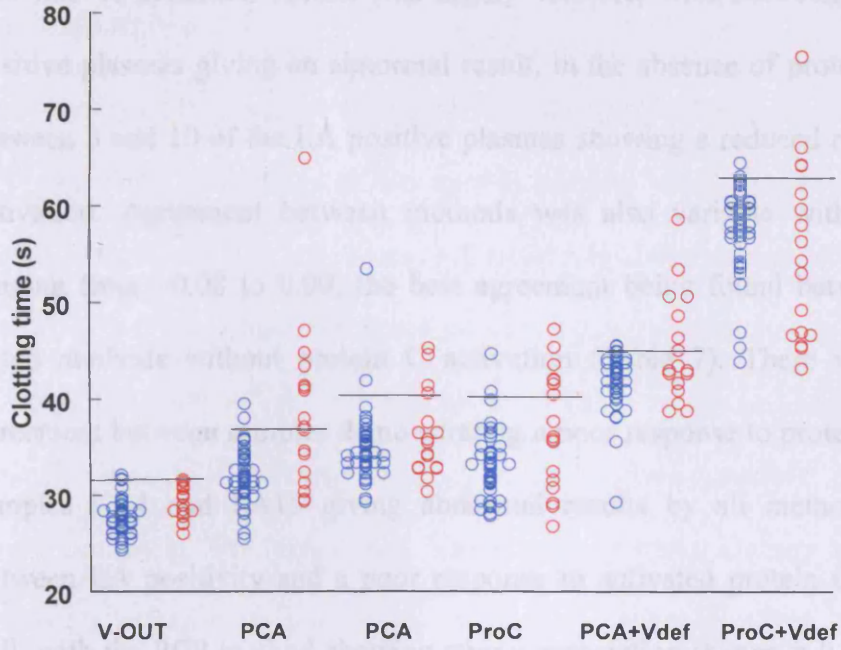

Figure 14: Raw clotting times for normal (blue) and LA positive (red) plasmas in the absence of protein C activation.
(The solid horizontal lines represent the 97.5th percentile for each method)

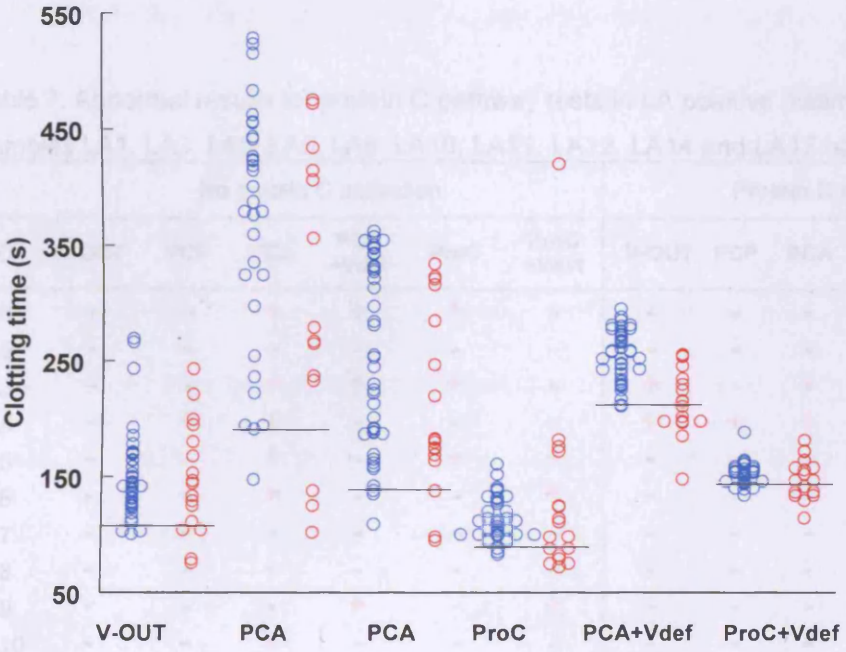

Figure 15: Raw clotting times for normal (blue) and LA positive (red) plasmas with protein C activation.
(The solid horizontal lines represent the 97.5th percentile for each method).

The rate of abnormal results was highly variable, with between 0 and 9 of the LA positive plasmas giving an abnormal result, in the absence of protein C activation, and between 3 and 10 of the LA positive plasmas showing a reduced response to protein C activation. Agreement between methods was also variable with kappa coefficients ranging from –0.08 to 0.90, the best agreement being found between the two APTT based methods without protein C activation (Table 7). There was more consistent agreement between samples demonstrating a poor response to protein C activation, with samples LA4 and LA15 giving abnormal results by all methods. The association between LA positivity and a poor response to activated protein C was more variable still, with the PCP method showing strong association (kappa = 0.82), the V-OUT and PCA test showing little or no association and the ProC test demonstrating a negative association with and without predilution in factor V deficient plasma (kappa = -0.42).

Table 7: Abnormal results for protein C pathway tests in LA positive plasmas (Positive dRVVT). Samples LA1, LA3, LA5, LA6, LA9, LA10, LA11, LA12, LA14 and LA17 had prolonged APTTs

	No protein C activation						Protein C activator added					
ID	V-OUT	PCP	PCA	PCA +Vdef	ProC	ProC +Vdef	V-OUT	PCP	PCA	PCA +Vdef	ProC	ProC +Vdef
LA1	-	-	+	+	+	-	-	-	-	-	-	-
LA2	-	-	-	-	-	-	-	-	-	-	+	-
LA3	-	-	+	+	+	-	+	-	+	+	-	-
LA4	-	+	+	-	-	-	+	+	+	+	+	+
LA5	-	-	+	-	+	+	-	-	-	-	-	-
LA6	-	-	+	-	-	+	-	-	-	+	-	-
LA7	-	-	-	-	-	-	-	-	-	+	+	+
LA8	-	-	-	-	-	-	-	-	-	+	-	+
LA9	-	-	-	+	-	+	-	-	-	+	-	-
LA10	-	-	+	-	-	-	-	-	-	-	-	-
LA11	-	-	+	+	+	+	-	-	-	+	-	-
LA12	-	-	-	-	-	-	-	-	-	-	+	-
LA13	-	-	-	-	-	-	-	-	-	-	-	-
LA14	-	-	+	-	+	-	+	-	-	-	-	-
LA15	-	+	+	-	-	-	+	+	+	+	+	+
LA16	-	+	-	+	+	-	-	+	-	+	+	-
LA17	-	+	+	+	+	-	+	-	+	+	-	+

3.4 Discussion

Four methods, designed to screen for defects in the protein C pathway were studied. 2.5^{th} and 97.5^{th} percentiles of the normal samples were used to determine normality. Three samples, which gave values well below the 2.5th percentile by all methods, were excluded from the normal reference range. Two were from women using combined oral contraceptives, while the other had an acute phase response. High factor VIII:c levels (Chitolie et al. 2001), characteristic of an acute-phase reaction, and/or low protein S levels, associated with pregnancy, hormone replacement therapy and certain combined oral contraceptives (Rosing et al. 1997), are known to affect APC ratios, possibly leading to misdiagnosis.

One method used both exogenous APC and activation of endogenous protein C (V-OUT), while the other methods rely exclusively on the activation of endogenous protein C. The V-OUT and ProC methods showed no significant differences between results for male and female normal subjects, whereas the PCA and PCP methods, males gave significantly higher values than females and, consequently, sex-specific cut-off ratios were employed. The two APTT based methods were also performed following predilution of plasmas with factor V-deficient plasma, and this produced a narrower spread of results. The addition of factor V-deficient plasma did not eliminate the sex-specific differences as may have been expected.

Detection of APCR due to the factor V Leiden mutation is prerequisite for any method intended to assess the protein C pathway, and all factor V Leiden positive samples were correctly identified by all methods. However, the degree of separation between normal subjects and samples from patients with factor V Leiden was variable. Predilution of the test samples in factor V-deficient plasma produced clearer separation between normals, heterozygotes and homozygotes, but as would be expected, massively reduced the

sensitivity to protein C and S. The three methods relying on the activation of endogenous protein C alone achieved 55–73% sensitivity to protein C deficiency, whereas the method using additional exogenous APC detected protein C deficiency in less than 30% of the plasmas tested. None of the methods were able to consistently identify protein S deficiencies, with sensitivities ranging from 11 to 47%.

Some plasmas from patients with thrombophilia, as defined by BCSH guidelines (Greaves et al 2001), but no evidence of protein C deficiency, protein S deficiency, lupus anticoagulant or factor V Leiden, gave low ratios with different methods. It was of interest that one patient, who had no known defect associated with thrombophilia, had low ratios by all three Protac®-based methods. This was in line with recent reports suggesting that 'Global tests' for thrombophilia may identify patients with yet undefined risk factors for thrombosis (Grand'Maison et al. 2005; Robert et al. 1999). As my work progressed, I discovered several possible mechanisms for acquired APC resistance, and, during this time the scientific community started to regard acquired resistance to APC as more than an 'artefact' or 'false positive' result (Koenen et al. 2003; Liestol et al. 2007; Robertson et al 2006; Tans et al. 2003). The mechanisms responsible came to dominate my thesis and are discussed in detail in the later chapters.

All four methods demonstrated excellent sensitivity for factor V Leiden when suitable cut-off values for normality were applied. However, all methods showed poor sensitivity for protein S deficiency, and only moderate sensitivity for protein C deficiency. Previous studies of some of these tests have demonstrated poor sensitivity to protein S deficiency, but have reported better detection rates for protein C deficiency (Haas, Sterkenburg-Kamp, & Scheepers 1998; Ruzicka et al. 1997; Toulon et al. 2000; Tripodi et al. 1998). There are several possible causes for the differences between this study and previous reports. I employed sex-specific protein S reference ranges (using healthy males and females, not known to be receiving drugs or hormones thought to

affect coagulation tests), which in several cases have resulted in a diagnosis of protein S deficiency at levels in excess of the 0.70 IU/ml cut-off used in many of these studies. Pre-analytical variables are also of prime importance in APCR testing; in our present study, all samples were double centrifuged at room temperature and stored at < 70°C for no longer than 12 months. Frozen plasma samples were thawed in a waterbath at 37°C immediately prior to use and were not refrozen. It has been shown that APCR testing is exquisitely sensitive to residual platelet contamination of plasma (Taube et al. 1999) and that clotting tests are affected by cold activation (Czendlik, Lammle, & Duckert 1985; Palmer & Gralnick 1982) yet, in several of the previous studies, plasma was prepared by a single cold centrifugation step. Furthermore, it has recently been reported that protein S is unstable in plasma stored at -24°C (Woodhams et al. 2001) and that storage below -70°C is advisable, yet in the largest of the published studies some samples were stored at -30°C. It was clear that these methods lacked sensitivity to protein C and protein S deficiencies, and although, they were suitable for the exclusion of factor V Leiden, providing that predilution in factor V-deficient plasma was used, they lacked specificity for factor V Leiden and could not replace the need for confirmation by PCR

In most of the published studies of methods for the assessment of the protein C pathway, testing for aPA was not performed, yet these antibodies are known to interfere with global clotting tests and protein C/protein S activity assays and are an important cause of the APCR phenotype in the absence of factor V Leiden (Bokarewa et al. 1994; Malia et al. 1990; Marciniak & Romond 1989). Consequently, it came as no surprise when I found low ratios in 18–35% of LA-positive plasmas. As would be expected, in some cases abnormal ratios were due to prolongation of the basal clotting time without protein C activation, due to lupus anticoagulant activity. However, there were striking differences between methods, with one (V-OUT) producing no abnormal basal clotting

times at all, and between four and ten prolonged clotting times using the other three methods. There was some agreement between the two APTT based methods, but it was abundantly clear that different plasmas caused prolongation of different methods. This is in keeping with previous studies at this centre, which confirmed that no single test is capable of detecting all LA (Gardiner et al 2000; Lawrie et al. 1999). Predilution in factor V deficient plasma failed to correct the prolonged clotting times in 10/17 cases, indicating that a potent inhibitor of in vitro coagulation was present.

However, it was clear that prolongation of the basal clotting times was not enough to explain the abnormally low ratios observed. A failure of the endogenous and/or exogenous APC to prolong the clotting time was observed in 13/17 plasma samples. Again, this finding was not consistent between methods, but good agreement was observed between the two viper venom based assays. Somewhat surprisingly, this effect was more apparent when the plasmas were prediluted in factor V deficient plasma. I looked for an association between the LA activity apparent in the screening test and the poor anticoagulant response to APC, but while this was apparent using one of the viper venom tests (PCP), there was no association for the other tests. Indeed, the converse appeared to be true for the ProC Global assay.

While it was clear that APC resistance was associated with LA in some patients, this was not a consistent finding. Although I knew little about the specific composition of the individual reagents, it was clear that the V-OUT method used either a high concentration of phospholipid, a 'lupus insensitive' phospholipid, or a combination of the two, as the basal clotting time was not prolonged by any of the seventeen LA positive plasmas tested. Yet, six of these plasmas demonstrated resistance to APC. While I had not demonstrated that aPA were responsible, the fact that predilution increased the resistance to APC strongly suggested that an inhibitor was responsible for

this phenomenon. As the plasmas were all from patients with APS, it seemed reasonable to attribute the inhibition of APC to an aPA; at least as a working hypothesis.

In the development of these 'protein C pathway' screening tests, the manufacturers' attempted to attenuate the "artefacts" caused by aPA, by using high concentrations of phospholipid, 'lupus insensitive' phospholipid and mixing with factor V depleted plasma. During the course of this investigation it occurred to me that, rather than viewing acquired APC resistance as an inconvenient artefact, I should investigate this phenomenon as a possible marker for the thrombotic disease in APS and the potential role of aPA interference in the protein C pathway in the pathogenesis of APS. The paradoxical association between the prolonged clotting time caused by LA and thrombosis has puzzled me, and others, for many years. If aPA were somehow responsible for acquired APC resistance, then this seemed to me a much more plausible explanation for their mode of action than prolonging clotting times.

In order to investigate this further, I devised methods for measuring the anticoagulant response to APC, which were sensitive to aPA so I aimed to investigate whether this phenomenon was antibody dependent and, if so, study the specificity of the antibodies responsible.

Chapter 4. The Detection of Acquired APC Resistance using a novel clotting test

4.1 Introduction

There is a great deal of heterogeneity of antibody populations, concentration and avidity amongst patients with aPA and not all of the target antigens are represented by the antibodies detected by the typical clinical laboratory. As discussed in the previous chapter, plasma from some patients with APS interferes with the protein C pathway and this may manifest as APC resistance in the absence of the factor V Leiden mutation (Aznar et al. 1997; Malia et al 1990; Marciniak et al 1989; Rosing et al. 2004; Sarig et al. 2002; Tans et al 2003). Consequently, a number of approaches have been used in attempts to attenuate this effect and to improve the specificity for factor V Leiden, including dilution in FV deficient plasma (Ivey et al. 2000; Le et al. 1995), use of reagents containing concentrated phospholipid (Haas et al 1998) or mixing patient plasma with normal plasma prior to testing (Jacobsen & Wisloff 1997).

It is known that APCR, even in the absence of factor V Leiden, is a risk factor for venous thromboembolic disease (VTE), both with (Male et al. 2001) and without aPA (de Visser, Rosendaal, & Bertina 1999). As most coagulation reactions require anionic phospholipids, in particular phosphatidyl serine (PS), and interference with anionic phospholipids is associated with LA, the importance of other phospholipids is frequently overlooked in this setting. However, the inactivation of factor Va is greatly enhanced by the presence of zwitterionic phospholipids, including phosphatidylethanolamine (PE) (Smirnov et al 1994), glucosylceramide (Yegneswaran, Deguchi, & Griffin 2003) and sphingomyelin (SM) (Deguchi, Fernandez, & Griffin 2002; Pecheniuk 2003). Cardiolipin, which is a component of the normal plasma lipoprotein fraction (Deguchi et al 2000), also enhances the anticoagulant function of the protein C pathway at physiological levels of cardiolipin (Fernandez et al 2000).

There have been several reports of "antibodies to phosphatidylethanolamine" as the only antiphospholipid antibodies, in patients with unexplained thrombosis (Sanmarco et al. 2001) (Berard et al. 1996) and vascular cutaneous diseases (Karmochkine et al. 1992), although it is now known that these antibodies are directed against phosphatidylethanolamine bound proteins of the kallikrein/kinin system (Sugi & Mclntyre 1996); (Katsunuma et al. 2003; Sugi et al 1996). Furthermore, it has been shown that some lupus anticoagulant activity can be inhibited by hexagonal phase phosphatidylethanolamine in the presence of prothrombin (Rauch et al. 1998).

I used these phenomena to develop a highly sensitive test for aPA-associated APCR, with the intention of investigating the incidence of acquired APC resistance. As previously mentioned, currently available APCR tests are designed to be insensitive to aPA and so I used a dilute phospholipid preparation, of known formulation, containing both phosphatidylethanolamine and phosphatidylserine, which I predicted would be sensitive to aPA interference in the protein C pathway. The test was, in essence, a modified Russell's viper venom time performed with and without endogenous protein C activation in the presence of rate limiting amounts of phospholipid. I hoped in this way, that I could get a better indication of the prevalence of acquired APC resistance and possible contributing mechanisms responsible. As no exogenous protein S was added to the assay, the effect of free protein S antigen was studied, as I suspected that this might be a confounding variable.

4.2 Methods

4.2.1. Patients and blood samples

Blood was collected and plasma prepared as described in the Methods chapter. Forty-four patients with aPA referred to the UCLH thrombosis outpatient clinic were included in the present study (eight men and 36 women, mean age 41.7 years, age range 17–74 years). All had LA, anti-β_2GPI, and/or aCL on two or more occasions at least 6 weeks apart. None of the patients had an acute thrombotic event, were receiving anticoagulants or were known to be pregnant at the time of testing. Their laboratory data are summarized inTable8. 32% of patients were positive for lupus anticoagulant alone, 36% had raised aCL titres alone, while 33% demonstrated both lupus anticoagulant and aCL positivity. 28% displayed positivity for anti- β2GPI, of which half also had LA activity. Forty-two of the aPA positive patients had no underlying autoimmune disease, while two patients had aPA that were secondary to systemic lupus erythematosus (SLE). Twenty-four had a history of thrombosis (twelve patients had venous thromboembolism, seven had arterial stroke, three had cerebral ischaemia secondary to arterial disease, and two had transient ischaemic attacks). Ten women had a history of aPA-associated pregnancy morbidity; three had early fetal loss (< 20 weeks) and seven had late obstetric complications (intrauterine fetal death, placental insufficiency, or premature birth due to preeclampsia). The remaining 10 aPA-positive patients had immune thrombocytopenic purpura (n = 3), migraine (n = 3), SLE (n = 2) or were asymptomatic with aPA found coincidentally following routine coagulation testing (n = 2).

A control group of 20 patients attending the haematology outpatient clinic (three men and 17 women, mean age 41.1 years, age range 14–68 years), who were negative for aPA or protein C pathway defects but with a similar clinical history were also studied. Nine of these patients had venous thromboembolism, seven had arterial stroke, four

suffered recurrent early foetal loss and one had an intrauterine death. An additional control group (factor V Leiden controls) of ten patients recruited from the haematology outpatient clinic (four men and six women Mean age 43.2 range 23 – 63 years), negative for aPA, but heterozygous for the factor V Leiden mutation were studied for comparison purposes. The normal reference range was established in 28 healthy normal volunteers (fourteen males and fourteen females, mean age 35.8 years, range 19 –51).

Table 8: Characteristics of the antiphospholipid positive patients studied: Median value and ranges shown. dRVVT shown was with dilute phospholipid in the screening part of the test. *According to the modified Sapporo criteria (Miyakis et al. 2006)

	dRVVT ratio	ACL IgG (GPLu)	ACL IgM (MPLu)	Anti-β_2 GPI IgG (GPLu)	Protein S IU/ml	Protein C IU/ml	APTT APCR
All aPA (n=44)	1.21 (0.88-2.39)	2.35 (0.0-92.2)	2.95 (0 -48.6)	1.1 (0.0-120.0)	1.00 (0.57-1.43)	1.12 (0.78-1.55)	2.28 (1.75-2.98)
APA pos thrombosis (n=24)	1.21 (0.88-2.39)	1.3 (0.0-92.2)	4.10 (0 -48.6)	1.2 (0.0-120.0)	1.05 (0.62-1.43)	1.23 (0.81-1.55)	2.28 (2.05-2.98)
APA pos pregnancy morbidity (n=10)	1.22 (1.01-1.55)	9.3 (0-86.1)	4.2 (0-38.8)	1.2 (0.7-19.2)	0.86 (0.43-1.14)	0.96 (0.57-1.19)	2.31 (1.75-2.65)
Definite APS* (n=23)	1.25 (0.63-2.39)	8.0 (0.0-92.2)	2.4 (0-48.6)	1.3 (0.0-75.0)	1.00 (0.43-1.24)	1.11 (0.57-1.55)	2.31 (1.75-2.76)
Reference range	< 1.13	< 5.0	<5.0	< 3.0	Male 0.73 – 1.76 Female 0.58 – 1.14	0.70 – 1.30	> 2.0

4.2.2 Endogenous APC ratio (EAPCR)

Acquired resistance to APC was assessed by the degree of prolongation of the clotting time, produced by activated endogenous protein C in a modified dRVVT with dilute phospholipid using RVV and Protac® as described in the methods chapter. Two phospholipid preparations were used: Bell and Alton platelet substitute (Diagnostic Reagents Ltd), and Actin FS (Dade Behring), which is 'lupus insensitive' (Brancaccio et al 1997; Lawrie et al 1998).

4.2.3 Statistical analysis

Due to the skewed nature of the data, non-parametric tests were used throughout. Normality was defined by the 2.5^{th} and 97.5^{th} percentiles of the 28 normal subjects and statistical significance was determined by the Wilcoxon signed rank test or Wilcoxon-Mann-Whitney U test, as $p < 0.05$.

4.3 Results

4.3.1 The effect of phospholipid concentration

The effect of phospholipid concentration on clotting times with and without endogenous protein C activation was studied. Normal pooled plasma was tested at a range of phospholipid reagent concentrations (doubling dilutions from 5 – 11,000 ng/mL). In order for the test to be sensitive to aPA interference in the procoagulant and protein C pathways, the phospholipid concentration had to be the rate-limiting step. 1375 ng/mL of the antiphospholipid was required to produce a demonstrable prolongation of the baseline clotting time. However, at this phospholipid concentration, the anticoagulant response to APC action was severely impaired (Figure 16). It was found that 5.5 μg/mL of the Bell and Alton platelet substitute gave optimum conditions, i.e., Protac significantly prolonged the clotting time while all plasma samples clotted within 300 seconds, thus allowing automated detection. This dilution was subsequently used throughout this study.

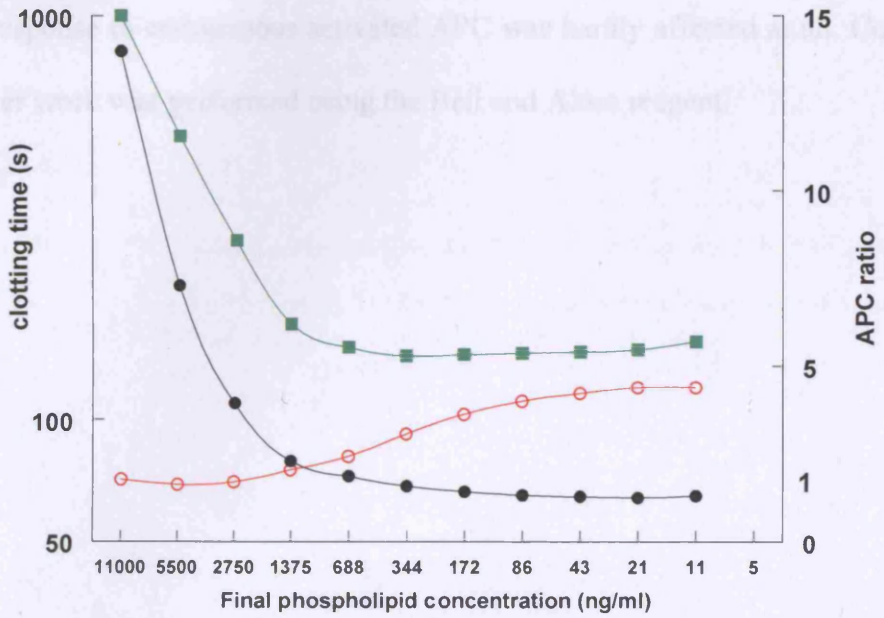

Figure 16 Effect of phospholipid concentration on RVV clotting time.
Normal plasma with Protac® ■, without Protac® ○, and the endogenous APC ratio (EAPCR) ●.

4.3.2 The effect of phospholipid composition

A reference range for the normalised endogenous activated protein C ratio (EAPCR) of 0.81-1.21 (95% confidence interval) was established in 28 normal subjects (median 1.04) with no significant difference between the sexes using the Bell and Alton reagent. Similar values were observed using the lupus insensitive reagent, (Actin FS; 0.72-1.27, median 0.97). However, the response to APC was much greater with the Bell and Alton reagent than the lupus insensitive reagent (Figure 17, Table 9) with mean Protac clotting time: clotting time (without Protac) ratios of 5.2 (SD = 0.6) and 2.7 (SD = 0.5) respectively. Forty of the 44 aPA-positive patients (91%) produced a high EAPCR, and this was predominantly due to a poor response to APC (33/40) rather than prolonged basal RVV clotting times alone (5/38) (Table 10, Figure 17). The same tests performed using the 'lupus-insensitive' phospholipid gave an abnormal result in only 16/44 (36%) of the aPA-positive patients. The second reagent was relatively insensitive to endogenous APC as judged by the poor anticoagulant response obtained by the action of Protac (Table 10). Furthermore, both limbs of the test appeared to be only mildly affected by aPA, i.e., the baseline clotting time was only slightly prolonged by aPA and the response to endogenous activated APC was hardly affected at all. Consequently, all further work was performed using the Bell and Alton reagent.

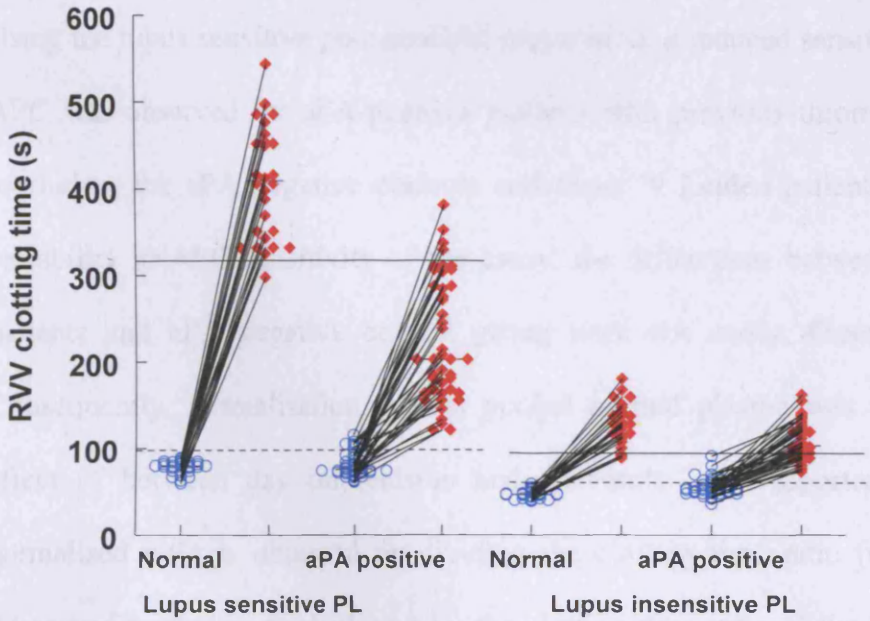

Figure 17 Effect of aPA on RVV clotting times
Clotting times with ◆ and without the addition of Protac® O, using a lupus anticoagulant sensitive, and a lupus anticoagulant insensitive phospholipid. The broken line is the 97.5th percentile of RVV clotting times without Protac® for the normal subjects. The unbroken line is the 2.5th percentile of RVV clotting times with Protac® for the normal subjects.

Table 9: Summary of statistical analysis of clotting times in normal plasma and phospholipid preparation (n = 28)
BA = lupus anticoagulant sensitive reagent (Bell & Alton). AFS = Lupus anticoagulant insensitive reagents (Actin FS). IQR = inter quartile range

	BA	BA + Protac	AFS	AFS Protac
Median	80.0	413.0	46.4	127.7
IQR	74.9 -82.9	355.3 -459.8	42.7 – 49.3	112.6 – 141.2
Minimum	59.5	297.0	39.4	89.2
Maximum	88.8	543.0	59.5	180

Table 10: Summary of statistical analysis of clotting times in patients with aPA and phospholipid preparation (n = 44)
BA = lupus anticoagulant sensitive reagent (Bell & Alton). AFS = Lupus anticoagulant insensitive reagents (Actin FS). IQR = inter quartile range

	BA	BA + Protac	AFS	AFS + Protac
Median	46.4	127.7	51.8	103.4
IQR	42.7 - 49.3	112.6 - 141.2	48.7 -58.0	90.5 - 124.2
Minimum	39.4	89.2	35.6	73.1
Maximum	59.5	180.0	110.6	162.0

4.3.3 Endogenous APC ratio (EAPCR) measurement

Using the lupus sensitive phospholipid preparation, a reduced sensitivity to endogenous APC was observed in: aPA-positive patients with previous thrombosis or pregnancy morbidity, the aPA negative controls and factor V Leiden patients. However, due to variability in APC sensitivity of the assay, the differences between the aPA positive patients and aPA negative control group were not easily discernable (Figure 18). Consequently, normalisation against pooled normal plasma was used to reduce the effect of between day imprecision and all results were reported as EAPCR. This normalised ratio is obtained by dividing the clotting time ratio (without Protac/with Protac) of pooled normal plasma by the clotting time ratio of the test sample, so that increasing EAPCR indicates increased resistance to APC.

The EAPCR in the 44 aPA-positive patients was significantly higher than the normal individuals (median 1.87 v 1.04; $P < 0.0001$) or the aPA-negative control group (median 1.14; SD, 0.19) (Figure19). aPA-positive patients with previous thrombosis and pregnancy morbidity groups had similar EAPCR values, with slightly higher values in the factor V Leiden group (Table 11). Both of the SLE patients, 2/3 ITP patients, 2/3 migraine patients and 1/2 coincidental aPA patients had a high EAPCR. When only patients fulfilling the criteria for definite APS (Miyakis et al 2006) were studied (n = 23), all had a high EAPCR (median 2.15; range 1.46 – 3.17).

Table 11: Summary of statistical analysis of EAPCR by patient group

	Normal (n = 28)	aPA +ve thrombosis (n = 26)	aPA +ve pregnancy morbidity (n = 24)	aPA negative control (n = 20)	Factor V Leiden (n = 10)
Median	1.04	1.80	2.27	1.08	2.64
IQR	0.90 – 1.06	1.48 – 2.38	1.64 – 2.70	1.05 – 1.26	2.53 – 2.91
Minimum	0.83	1.18	1.29	0.79	2.14
Maximum	1.26	3.11	3.26	1.63	3.83

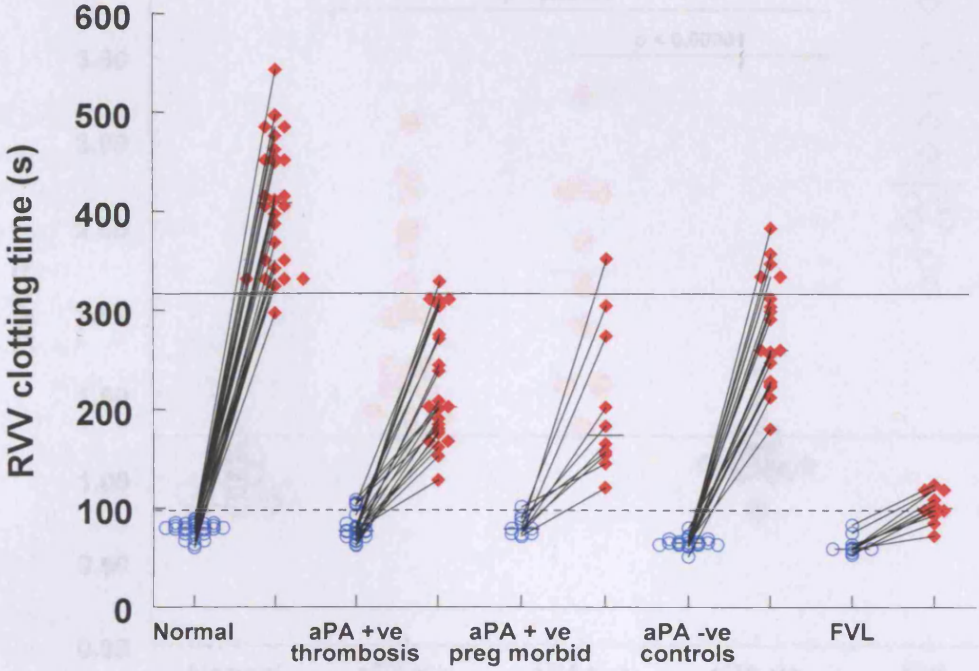

Figure 18: Effect of clinical group on RVV clotting times with ◆ and without the addition of Protac® ○, using a lupus anticoagulant sensitive phospholipid. The broken line is the 97.5th percentile of RVV clotting times without Protac® for the normal subjects. The unbroken line is the 2.5th percentile of RVV clotting times with Protac® for the normal subjects.

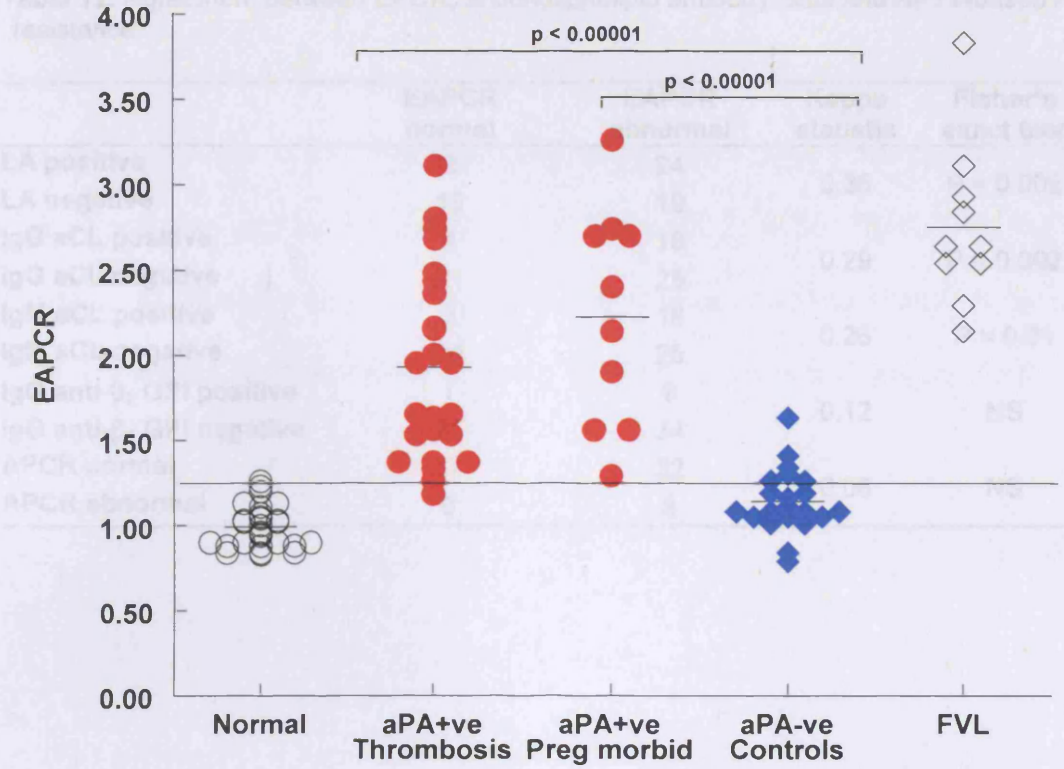

Figure 19: Endogenous APC ratio by clinical group.
Normal subjects (O), aPA positive patients (●) aPA negative control patients (◆) and patients with factor V Leiden (◇). The broken line represents the normal cut-off value (97.5th percentile).

Abnormal EAPCR showed significant association with LA, IgG aCL and IgM aCL, but no association with high IgG anti-β_2GPI or APTT based APC resistance (Table 12). This is due to the low incidence of abnormal IgG anti-β_2GPI levels or low APTT-based APC ratios, and the high incidence of abnormal EAPCR. The EAPCR value showed significant negative correlation with the APTT-based APC resistance method (Figure 20) ($r = -0.61$, $P = 0.001$). However, only three aPA-positive patients demonstrated APCR using the APTT-based method, compared with 41/44 for the EAPCR method. Predilution of plasma in factor V deficient plasma was not used in the determination of APTT-based APC resistance.

Table 12: Agreement between EPCR, antiphospholipid antibody tests and APTT-based APC resistance

	EAPCR normal	EAPCR abnormal	Kappa statistic	Fisher's exact test
LA positive	3	24	0.36	P = 0.002
LA negative	19	19		
IgG aCL positive	1	18	0.29	P = 0.002
IgG aCL negative	21	25		
IgM aCL positive	2	18	0.26	P = 0.01
IgM aCL negative	20	25		
IgG anti-β_2 GPI positive	1	9	0.12	NS
IgG anti-β_2 GPI negative	21	34		
APCR normal	17	32	0.06	NS
APCR abnormal	0	3		

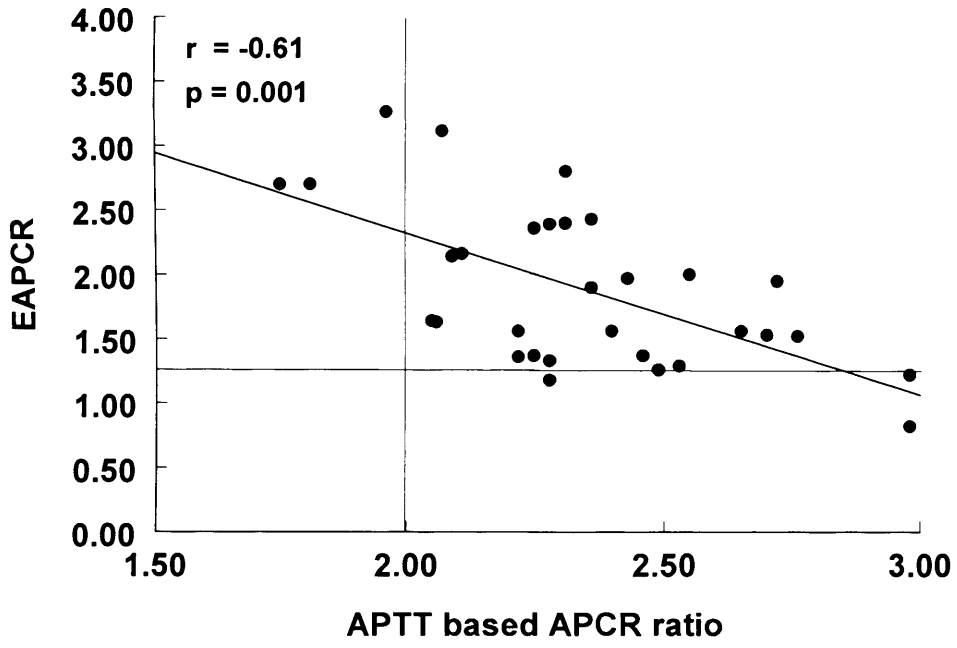

Figure 20: Endogenous APC ratio (EAPCR) v APTT based APC resistance ratio.
The broken lines represent the upper limits of normal for EAPCR and for the APTT based APC resistance method.

A significant correlation was observed between the EAPCR and dRVVT (Figure 21), and this remained when only LA positive patients were analysed (rs = 0.56, P = 0.0009). IgG anticardiolipin (Figure 22) but not IgM anticardiolipin (Figure 23) were significantly correlated with EAPCR. Although all but one of the patients with raised

IgG anti-β_2GPI levels also had a high EAPCR, the converse was not true, in that majority of patients with a high EAPCR did not have increased levels of IgG anti-β_2GPI and no significant correlation was observed (Figure 24). Free protein S antigen demonstrated a significant negative correlation with EAPCR (Figure 25), despite the exclusion of patients with protein S deficiency or those receiving vitamin K antagonists from this cohort. No relationship between the EAPCR value and protein C activity was observed.

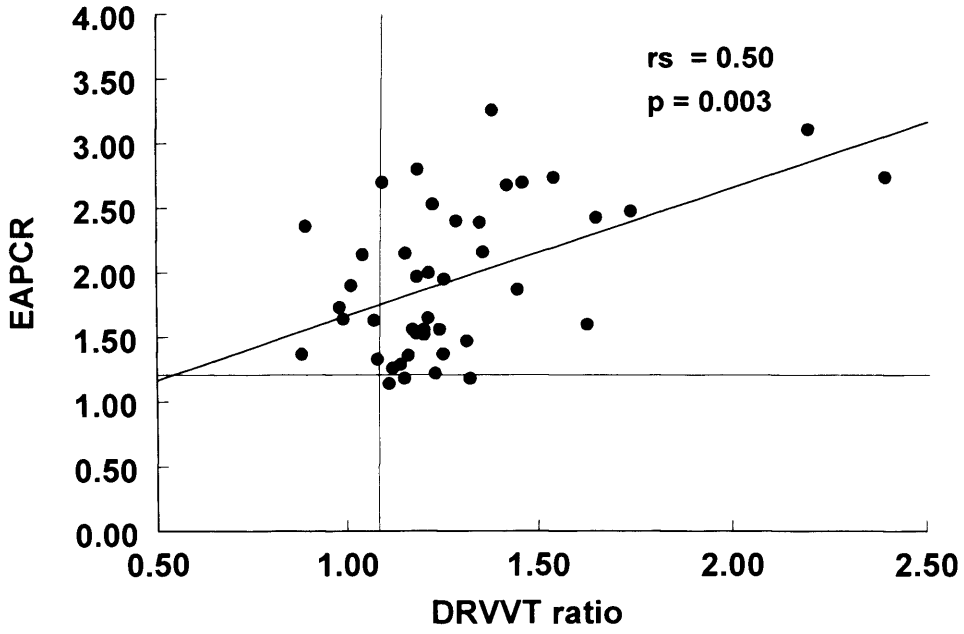

Figure 21: Endogenous APC ratio (EAPCR) v dRVVT ratio (using the dilute phospholipid in screening test).
The broken lines represent the upper limit of normal for EAPCR and the dRVVT ratio.

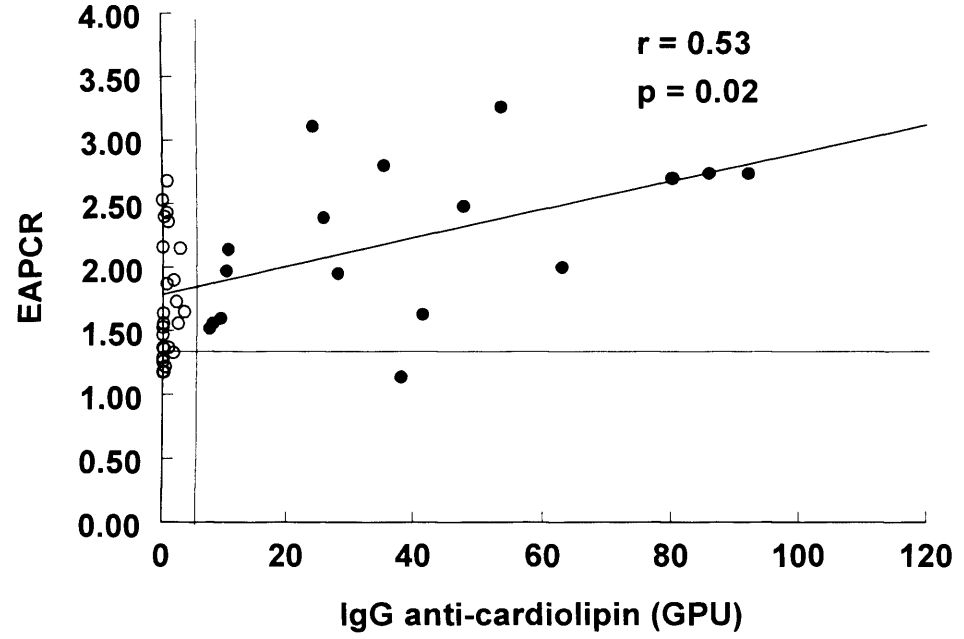

Figure 22 EAPCR v IgG anticardiolipin antibody concentration.
The broken lines represent the upper limit of normal for EAPCR and anticardiolipin. The regression line was performed on plasmas with IgG cardiolipin values of >5.0 GPU only.

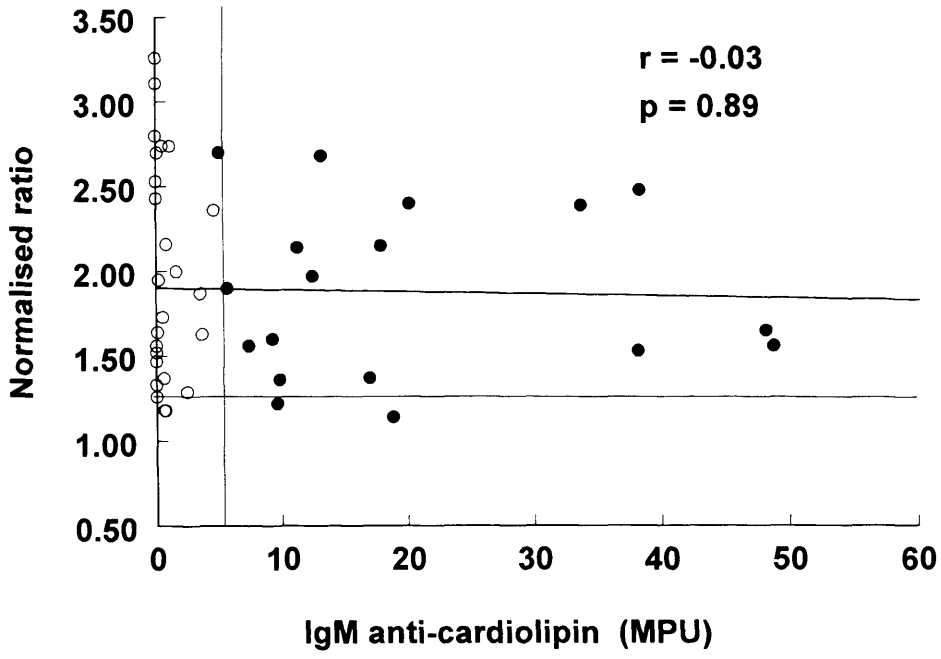

Figure 23 EAPCR v IgM anticardiolipin antibody concentration.
The broken lines represent the upper limit of normal for EAPCR and anticardiolipin. The regression line was performed on plasmas with IgG cardiolipin values of >5.0 MPU only.

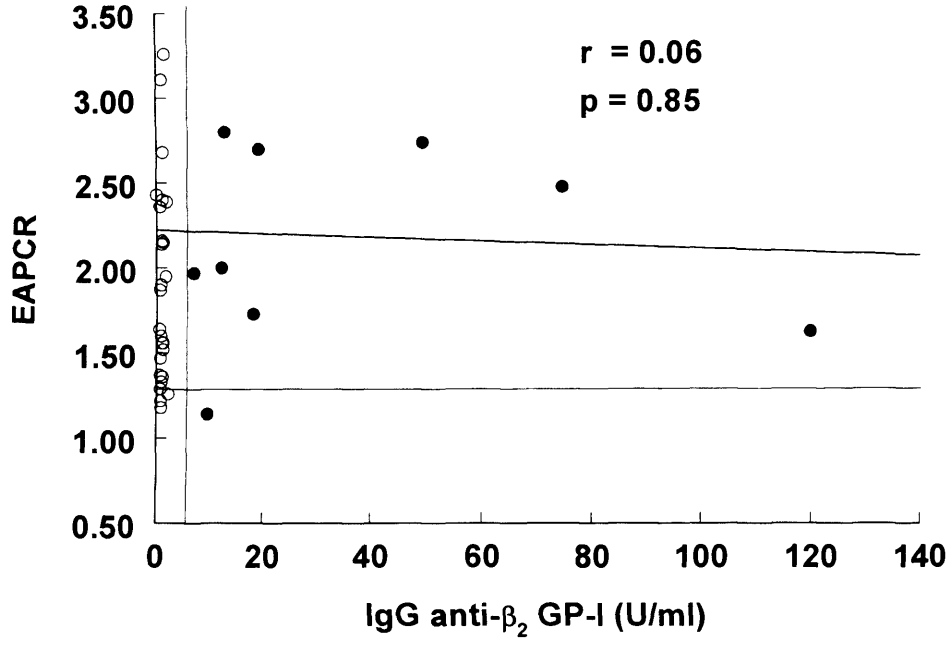

Figure 24. EAPCR v IgG anti-β_2 glycoprotein-I antibody concentration.
The broken lines represent the upper limit of normal for EAPCR and anti-β_2 glycoprotein-I. The regression line was performed on plasmas with IgG cardiolipin values of >5.0 U/ml only.

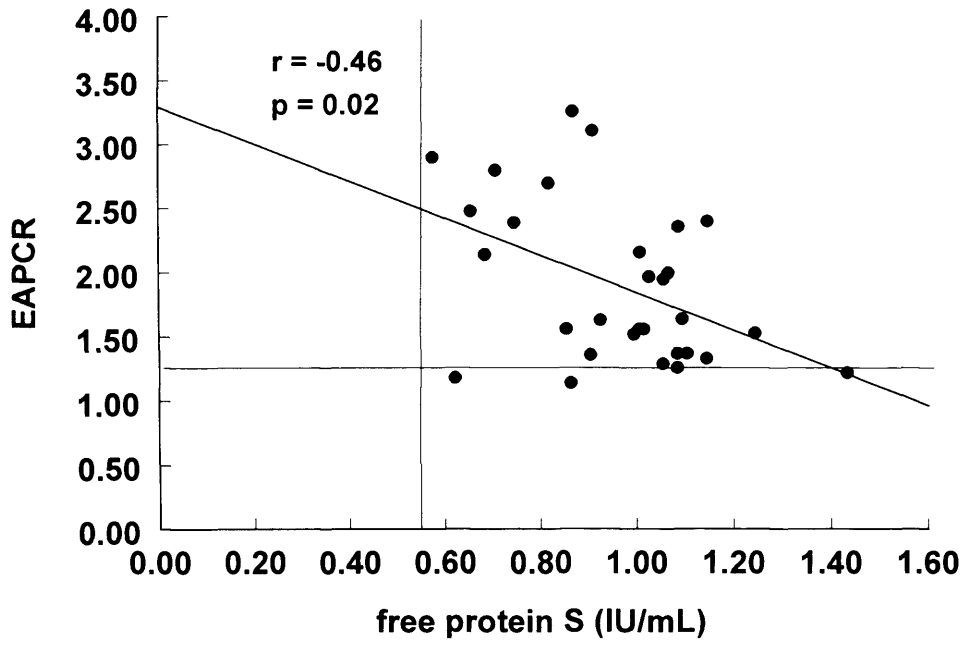

Figure 25 EAPCR v IgG free protein S concentration.
The broken lines represent the upper limit of normal for EAPCR and the lower limit for free protein S.

4.3.4 Effect of IgG fractions from patients with antiphospholipid antibodies

In order to ascertain whether the observed resistance to APC was due to the antiphospholipid antibodies rather than some other perturbation of the protein C pathway, IgG fractions were prepared from four healthy normal individuals and six patients with APS with a range of symptoms and antiphospholipid antibody types (Table 13).

Table 13: Characteristics of donors used for the IgG fractions

IgG fraction	Lupus anticoagulant	Anticardiolipin	Anti-β_2 Glycoprotein-I	Clinical history
N1	-	-	-	None
N2	-	-	-	None
N2	-	-	-	None
N4	-	-	-	None
aPA1	-	+	-	Stroke SLE
aPA2	+	+++	+++	VTE
aPA3	+	+	-	VTE
aPA4	++	+++	+++	Stroke LPP
aPA5	+++	-	-	Stroke SLE
aPA6	+	+	+	VTE

VTE = venous thromboembolism; SLE = systemic lupus erythematosus; LPP = late pregnancy problems, i.e. preeclampsia and intrauterine growth retardation

When the four normal IgG fractions at approximately 6 mg/ml were added to pooled normal pooled plasma (PNP), an increase of up to 5 seconds was observed in the RVV clotting time (Figure 26), causing a slight increase in the EAPCR (0–8%). The six IgG fractions from aPA patients showed a variable effect on the RVV clotting times at the same concentrations, with two of the six demonstrating LA activity by this method. However, all except one caused significant reductions in the RVV + Protac clotting time (Figure 26), and EAPCR (Figure 27), indicating that antibodies from patients with APS caused resistance to APC to varying degrees. It was notable that the IgG fraction causing the greatest amount of APC resistance (aPA5) was isolated from a patient without detectable anti-β_2GPI. When one of these IgG preparations (aPA6) was added to normal plasma at several dilutions, the increase in the EAPCR occurred in a dose-dependent manner (Figure 28).

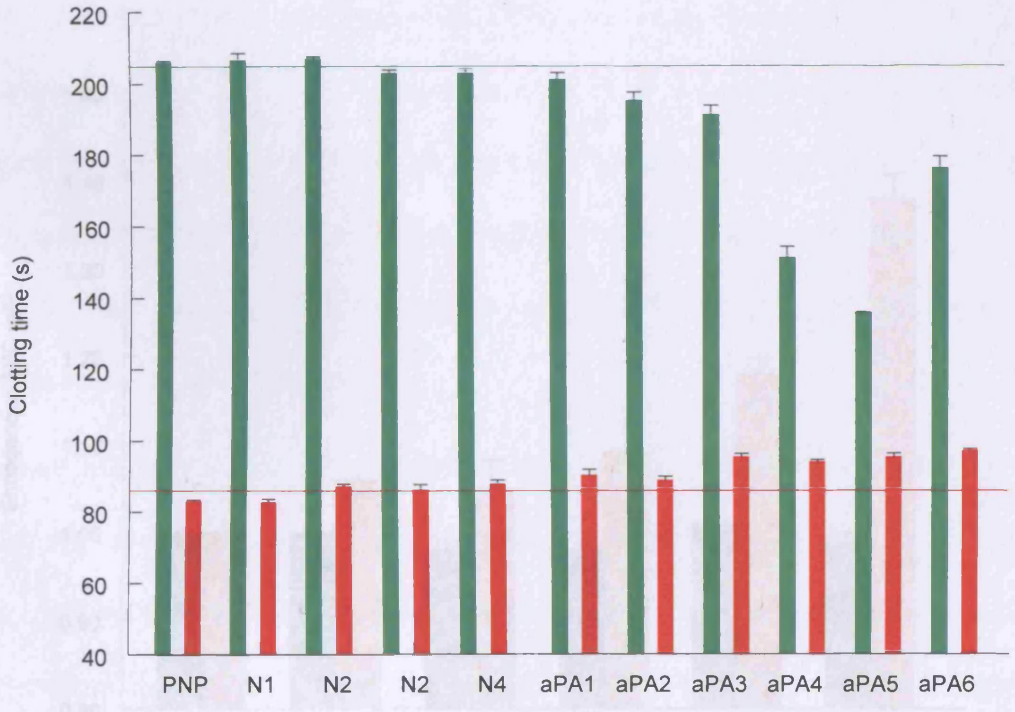

Figure 26 Effect of the addition of purified immunoglobulin

Immunoglobulin from normal subjects (N1 - N4) and patients with the antiphospholipid syndrome (aPA1 -aPA 6) on the RVV (■) and RVV + Protac(■) clotting times. The mean normal clotting times are indicated by the broken lines.

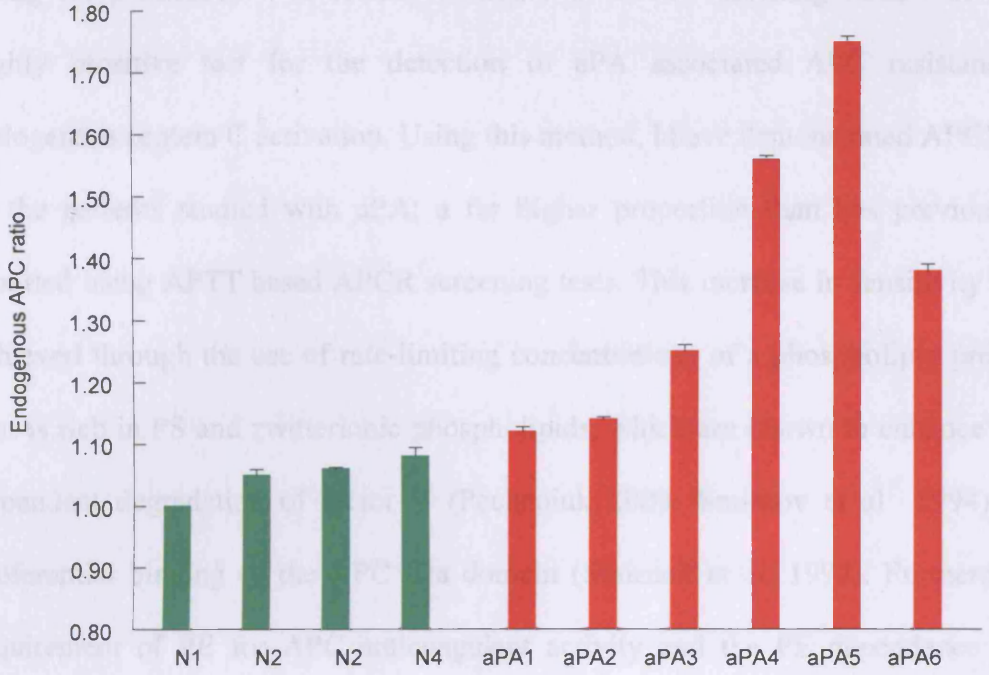

Figure 27 Effect of the addition of purified immunoglobulin
Normal subjects (N1 - N4 ■) and patients with the antiphospholipid syndrome (aPA1 –aPA 6 ■) on the endogenous APC ratio.

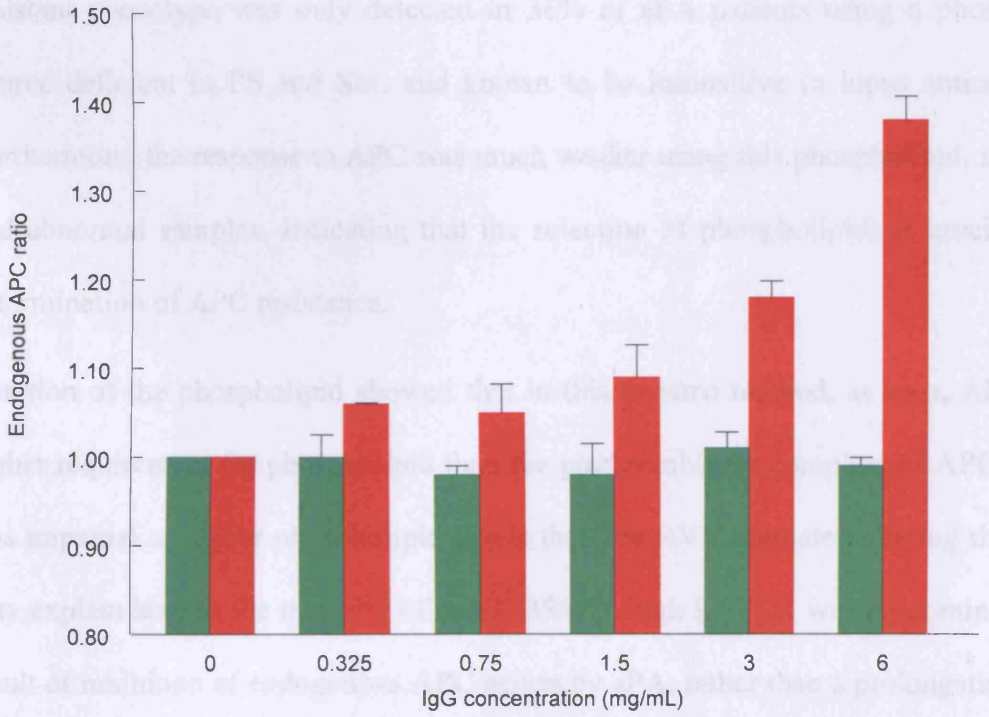

Figure 28 Dose response effect of added purified immunoglobulin
Pooled normal plasma (■) and a patient with the antiphospholipid syndrome (aPA 6 ■).

4.4. Discussion

Using the phenomenon of aPA interference in APCR screening tests, I developed a highly sensitive test for the detection of aPA associated APC resistance using endogenous protein C activation. Using this method, I have demonstrated APCR in 91% of the patients studied with aPA; a far higher proportion than has previously been reported using APTT based APCR screening tests. This increase in sensitivity has been achieved through the use of rate-limiting concentrations of a phospholipid preparation, that is rich in PS and zwitterionic phospholipids, which are known to enhance the APC dependent degradation of factor V (Pecheniuk 2003; Smirnov et al 1994) through preferential binding of the APC Gla domain (Smirnov et al. 1998). Furthermore, the requirement of PE for APC anticoagulant activity and the PE dependence of some antiphospholipid antibodies provide a basis for the selective inhibition of the protein C pathway in the pathogenesis of aPA (Balada et al. 2001; Berard et al 1996; Esmon et al. 2000; Sanmarco et al 2001; Sugi et al. 1999; Sugi et al. 2004). By contrast, the APC resistant phenotype was only detected in 36% of aPA patients using a phospholipid source deficient in PS and SM, and known to be insensitive to lupus anticoagulant. Furthermore, the response to APC was much weaker using this phospholipid, in normal and abnormal samples, indicating that the selection of phospholipids is crucial in the determination of APC resistance.

Titration of the phospholipid showed that in this *in vitro* method, at least, APC has a higher requirement for phospholipid than the prothrombinase complex, as APC activity was impaired at higher phospholipid levels than the RVV mediated clotting time. This may explain how in the majority of cases (33/40), high EAPCR was predominantly the result of inhibition of endogenous APC action by aPA, rather than a prolongation of the basal clotting time by LA activity.

High EAPCR was found in 96% (27/28) of patients fulfilling the modified Sapporo criteria for definite APS (Miyakis et al 2006). Only 3/20 of the aPA negative control group demonstrated high EAPCR (one had arterial stroke, one recurrent miscarriage and one VTE). The reasons for this are unclear, but APC resistance in the absence of factor V Leiden is known to be an independent risk factor for both thrombosis (de Visser et al 1999) and pregnancy morbidity (Rai et al. 1996). Furthermore, increases in plasma factor VIII:C (Chitolie et al 2001)) or prothrombin, and low levels of free protein S (de Visser et al. 2005)are also known to cause APC resistance. As may have been expected, the EAPCR showed a positive correlation with the APTT based APCR ratio, but only 3/42 (7.0%) aPA positive patients gave results below the normal cut-off value for the APTT based method, demonstrating the increased sensitivity of the EAPCR method. This was not surprising, since the APTT-based method is primarily intended as a screening test for factor V Leiden.

Despite the exclusion of patients known to be deficient in protein S (i.e., patients with known congenital deficiency or those receiving vitamin K antagonists), from this cohort of patients, free protein S showed a significant correlation with EAPCR. This indicates that free protein S concentration may be a confounding variable in this assay.

EAPCR showed weak negative correlation with dRVVT ratio and a highly significant negative correlation with IgG anticardiolipin, but not IgM anticardiolipin antibody levels. In several cases very low EAPCR (< 0.50), similar to those encountered in factor V Leiden patients, were observed in patients with a poor clinical history whose only previous abnormal finding was a persistently raised IgG anticardiolipin. This is consistent with the findings of Nash *et al* (2004), who showed that the anticardiolipin assay is required for sensitive screening for the antiphospholipid syndrome. Most patients with raised IgG anti-β_2GPI demonstrated resistance to APC as previously reported by Martinuzzo *et al* (1996) who reported that acquired APC resistance in

patients with aPL was associated with anti-β_2GPI rather than an *in vitro* interference by LA. However, the converse was not true, in that most patients with high EAPCR did not have raised levels of IgG anti-β_2GPI, which is contrary to the findings of Galli *et al* (1998) who reported that APC resistance was strictly -β_2GPI-dependent. (Galli, Ruggeri, & Barbui 1998). At the time of this part of my study, IgM anti-β_2GPI antibody assays were not readily available.

Using purified IgG from patients with APS, I was able to demonstrate that, in addition to the expected lupus anticoagulant effect, the antibodies interfered with the ability of activated endogenous protein C to prolong the RVV clotting time. This effect was highly variable, reflecting the heterogeneous nature of aPA, but the addition of increasing amounts of IgG caused acquired APC resistance in a dose response manner. Thus, I showed that the resistance to APC observed in these patients was an antibody-mediated effect, rather than a quantitative defect in the components of the protein C pathway. Two of the IgG fractions without anti-β_2GPI activity produced APC resistance, confirming that, while anti-β_2GPI antibodies may contribute towards APC resistance in some patients, anti-β_2GPI antibodies are not prerequisite for aPA associated resistance to APC.

There are several proposed mechanisms for aPA inhibition of the protein C pathway; Inhibition of thrombin formation leading to decreased protein C activation has been proposed as a possible mechanism but this is unlikely as high levels of thrombin favour coagulation rather than protein C activation (Griffin 1995), although this is dependent on the site of thrombin generation and the density of thrombomodulin present (Lane et al 2005). It has also been reported that antibodies may bind protein C and protein S directly (Oosting et al. 1993a; Oosting et al 1993b), possibly inhibiting the assembly of the protein C complex (Malia et al 1990). This may occur through direct interference with the residues responsible for APC-protein S binding or through the inhibition of

protein S binding to phospholipid, rather than the inhibition of Gla domain-dependent binding of APC to phospholipid *per se* (Preston et al. 2005).

It has been reported that aPA increase the affinity of C4b binding protein for protein S thus causing acquired deficiency of free protein S (Atsumi et al. 1997). Anti-β_2GPI strongly inhibits the binding of APC at concentrations which cause only weak inhibition of phospholipid-dependent prothrombinase activity (Mori et al. 1996) thus producing a net prothrombotic effect. However, β_2GPI binding to membranes with physiological anionic phospholipid content is relatively weak in comparison to plasma coagulation proteins (Harper et al. 1998) and this has led to speculation of the role of anti-β_2GPI antibodies in the pathology of aPA. It has been shown that β_2GPI is necessary for the inhibition of protein C activity by some monoclonal anticardiolipin antibodies (Ieko et al. 1999) and that this is achieved by increased binding to phospholipid surfaces facilitated by the dimerisation of β_2GPI by aPA molecules. (Takeya et al 1997). Given the heterogeneous nature of aPA, it is probable that more than one species of antibody is responsible for the inhibition of factor Va cleavage by APC. Anti-prothrombin has been shown to cause LA activity by a mechanism similar to that of β_2GPI (Simmelink et al 2001), and was recently reported to inhibit the inactivation of factor Va by activated protein C (Galli et al. 2005). It seems reasonable to suggest that anti-prothrombin could also cause aPA associated APCR by competition for phospholipid surfaces.

In conclusion, I demonstrated an APCR phenotype, rarely shown by conventional APTT-based APC resistance screening tests, in the majority of patients with aPA in the absence of FV Leiden. This was an antibody mediated effect, which was partially due to the LA activity prolonging the baseline clotting time, but the main effect was a significant inhibitory effect on the activity of endogenous APC. Having confirmed that APC activity has a higher requirement for phospholipid than that of the prothrombinase complex, it seemed reasonable to predict that this would result in a net prothrombotic

effect when aPA causes competition for phospholipid binding sites. I believe that this goes some way to explaining the 'lupus anticoagulant paradox'.

This study suggests that this could be a useful test in identifying APC resistance in APS. However, this was a retrospective study, from a single centre. Most patients referred to our haematology clinic have no underlying autoimmune disease, i.e., they have primary APS. Furthermore, the patients selected were positive for aCL and/or dRVVT on at least two occasions, and these factors may have contributed to a selection bias. Ideally, it would be useful to conduct a prospective study of newly referred patients with suspected APS, and conduct serial studies on the same patients. Unfortunately, like so many other LA tests and global clotting tests, this method is unsuitable for patients receiving oral anticoagulation or heparin therapy.

It was clear that further work was required to elucidate the precise mechanisms and significance of acquired APCR in aPA patients. The rapid technological developments in the measurement of thrombin generation, which were occurring during the course of this thesis, raised the possibility of investigating the prothrombotic effect of aPA using an endogenous thrombin potential (ETP) based APC sensitivity assay.

Chapter 5. The Development and characterisation of an *in vitro* thrombin generation assay to assess the protein C pathway

5.1 Introduction

Although antithrombin is the principal inhibitor of free thrombin, the protein C pathway is the major feedback mechanism to control the amount of thrombin generated in vivo. The protein C pathway also plays a major role in localising thrombin generation to the site of injury (Oliver et al. 2002), as APC does not regulate thrombin production on the platelet surface but acts on the vascular endothelium. Consequently, although excessive thrombin generation *per se* is strongly associated with hypercoagulable states, the amount of thrombin formed in the presence of APC is probably more significant than the total amount of thrombin generated in its absence, when determining thrombotic risk. This is particularly true of the APS, as it has been reported that overall thrombin generation is reduced in the majority of cases (Hanly & Smith 2000), which would be expected to cause a bleeding disorder rather than thrombosis.

With the addition of phospholipid vesicles and rhAPC, it is possible to measure the effect of the protein C system on amount of thrombin formed, or the APC sensitivity ratio (nAPCsr). This may be used to measure changes in sensitivity to APC caused by protein S deficiency, oral contraception and pregnancy (Nicolaes et al. 1997). The nAPCsr is reported to be a strong risk factor for venous thrombosis (Tans et al 2003).

In order to study thrombin generation in patients with aPA, I developed an automated ETP method that was sensitive to APC, with conditions as close to physiological concentrations as possible. It was important that the assay was as sensitive to as many recognised prothrombotic variables as possible. As APC has a minimal effect on thrombin generation on platelets (Oliver et al 2002), it was neither necessary nor desirable that the method used platelet rich plasma. However, it was necessary to provide a phospholipid surface for the inactivation of factor Va by APC.

In chapter 3, I showed that the existing methods for assessing the protein C pathway were insensitive to protein S deficiency. The Russell's viper venom method, discussed in chapter 4 showed an improvement in sensitivity to protein S, but I felt that it was important that the thrombin generation method that I was to use was more sensitive to fluctuations in protein S, especially as aPA interference with protein S had been implicated in the pathology of APS (Malia et al 1990).

I therefore used the thrombin generation method to investigate samples from a previous study of the combined oral contraceptive pill (COC) (Mackie et al. 2001), where decreased protein S levels had been observed in association with 3rd generation progestogens. COC use has been associated with a 3-6 fold increase in relative risk of venous thromboembolism (Vandenbroucke et al. 2001; World Health Organization 1998). It has previously been reported that 3rd generation COCs (3rd generation) may cause greater resistance to APC than 2nd generation COCs (2nd generation)(Rosing et al 1997; Simioni et al. 1996) and this has been proposed as a possible mechanism of the reported increased risk of VTE. I knew that the changes observed in protein S and sensitivity to APC were relatively small, so this represented an ideal opportunity to ascertain the sensitivity and potential utility of the method.

I also wanted to study the kinetics of thrombin generation in plasma from patients with APS, to ascertain whether this could explain the increased risk of thrombosis. It has been variously reported that aPA inhibit thrombin generation (Hanly et al 2000; Sheng et al. 2001a) or accelerate thrombin generation through disruption of annexin V binding (Rand, Wu, & Giesen 1999) or inhibition of TFPI (Adams et al. 2004).

The aims of this section of my thesis were:

- To use the knowledge that I obtained from the RVV based method to establish the optimal conditions for measuring APC resistance using a sub-sampling thrombin generation method
- To ascertain whether this technique could be useful in the investigation of the putative prothrombotic mechanisms of APS.
- To standardise this method and transfer it to an automated platform.
- To identify the key determinants of this assay, with particular reference to conditions causing acquired resistance to APC.

5.2 Methods and materials

5.2.1 Patients and samples

Sample collection was performed as described in the methods chapter. Normal reference ranges were established in 30 healthy normal subjects (20 females, 10 males; mean age 34 years). None of the normal subjects were using anticoagulants, combined oral contraceptives, or any other drugs known to affect the coagulation system, at the time of testing.

The influence of plasma prothrombin levels and free protein S on thrombin generation was studied in women receiving COCs. The effect of factor V Leiden, aPA and acquired TFPI deficiency were studied in plasma from patients referred to the haematology outpatient clinics at UCLH for investigation of thrombophilia or pregnancy morbidity. The effect of factor VIII concentration was performed by adding purified factor VIIII to plasma from a patient with severe haemophilia A. None of these patients were receiving oral anticoagulants or heparin at the time of testing.

5.2.1.2 Women receiving the oral contraceptive pill

In a previous study we investigated the impact of omitting the pill free interval (PFI) on a variety of haemostasis activation markers and inhibitors, and any differences in women receiving combined oral contraceptives (COCs) containing 30μg ethinyl estradiol and either levonorgestrel (2^{nd} generation) or desogestrel (3^{rd} generation). It was found that free protein S levels were lower in 3^{rd} generation than in 2^{nd} generation COC users. Furthermore, protein S levels decreased in women switching from 2^{nd} to 3^{rd} generation COCs and this was mirrored by an increase in protein S in women switching from 3^{rd} to 2^{nd} generation COCs. Against this background, I decided to investigate whether this decrease in protein S was associated with the reported increase in APC

resistance. As increased plasma prothrombin levels have also been reported to influence resistance to APC, this was also investigated

This was part of a larger prospective, non-randomised, comparative study previously reported (Mackie et al 2001). Due to limited sample volume, and to avoid the adverse effect of re-freezing and thawing samples, I investigated samples from a sub-set of 26 COC users randomly selected from the original study. These were healthy established users of 2nd or 3rd generation COCs. The women omitted one pill free interval (PFI), switching immediately either to the opposite formulation for one cycle, or continuing the same pill. The four subgroups were designated.

AA Remained on 2nd generation throughout (n=6)

AB Started on 2nd and switched to 3rd generation (n=7)

BB Remained on 3rd generation throughout (n=6)

BA Started on 3rd and switched to 2nd generation (n=7)

In subgroups AA and BB, after one monitored cycle of 21 pills followed by a seven day PFI, the women were asked to omit the next PFI. In subgroups AB and BA, after one monitored normal COC cycle and the PFI that followed the women were asked to omit the next PFI and start taking the opposite COC type immediately. Venesection followed the initial PFI, after 1 cycle (21 tablets) and 2 cycles (42 tablets) of continuous pill taking. These were designated sample I, sample II and sample III respectively.

5.2.2 Development of sub-sampling technique

Initially I concentrated on developing a manual sub-sampling technique, using a method based on those of Nicolaes *et al* (1997) and Rosing *et al* (1999). APC resistance was measured as a sensitivity ratio (nAPCsr) in an amidolytic assay of tissue factor initiated

thrombin generation, with and without exogenous rhAPC. Measurement of thrombin generation was performed in plasma defibrinated using Ancrod at 37°C.

Initial experiments used 1/4 diluted Innovin (Dade Behring) as a source of tissue factor and freshly prepared phospholipid vesicles. This resulted in consistent thrombin generation, but negligible APC activity at 10nM. Increasing the APC concentration had little effect (data not shown), and did not seem to be a reasonable option as this would involve plasma APC concentrations far in excess of those seen in vivo. I inferred that the absence of visible APC activity was most probably due to the rapid accumulation of FVa having a swamping effect on the APC.

I titrated Innovin to examine the effect of tissue factor concentration and found that by using a tissue factor dilution of 1:32 (final dilution 1/192) that a good anticoagulant response was achieved. The final concentration of tissue factor at this dilution was approximately 20 pM as determined using the IMUBIND® Tissue Factor ELISA (American Diagnostica Inc, Stamford, CT).).

Using these conditions, I could achieve an ETP^{-APC}/ETP^{+APC} ratio of approximately 2.5. Although the initial results were encouraging, the day to day imprecision of the method was unacceptably high (CV in the order of 25%). As the reconstituted rhAPC was stored in aliquots at -80°C in a buffer containing 1% albumin I deemed this an unlikely source of variation. Using Innovin within 8 hours of reconstitution partially eliminated the variation, as did the use of freshly prepared buffers (<14 days). I suspected that the remaining imprecision was due to oxidation of the phospholipid used to prepare the vesicles. This was overcome by using the phospholipid preparation described in methods chapter.

5.2.3 A thrombin generation-based APC sensitivity test using an automated coagulometer (ACL 9000).

In order to follow up these initial findings, it was necessary to screen a large number of patients with aPA using an APCR ETP method. The sub-sampling technique, while useful with small numbers of samples, was technically difficult and time consuming. I needed to develop an automated version of the same test. This was achieved by constant measurement of thrombin generation using a slow reacting thrombin substrate (Pefachrome TG) using an Instrumentation Laboratory ACL9000. To improve sensitivity to factor VIII, the tissue factor concentration was reduced to 7 pM (Innovin at a final dilution of 1/600). As reducing the tissue factor concentration slows the activation of factor V to factor Va, I was able to reduce the concentration of rhAPC to 5 nM. I also reduced the phospholipid vesicle concentration to 20 μM in order to increase sensitivity to aPA.

The final method used is described in full in the methods chapter. Results were displayed cumulatively (Figure 29) or as the amount of thrombin generation against time (Figure 30). This allowed the calculation of total thrombin generation, lag phase to the start of thrombin generation and peak thrombin generation.

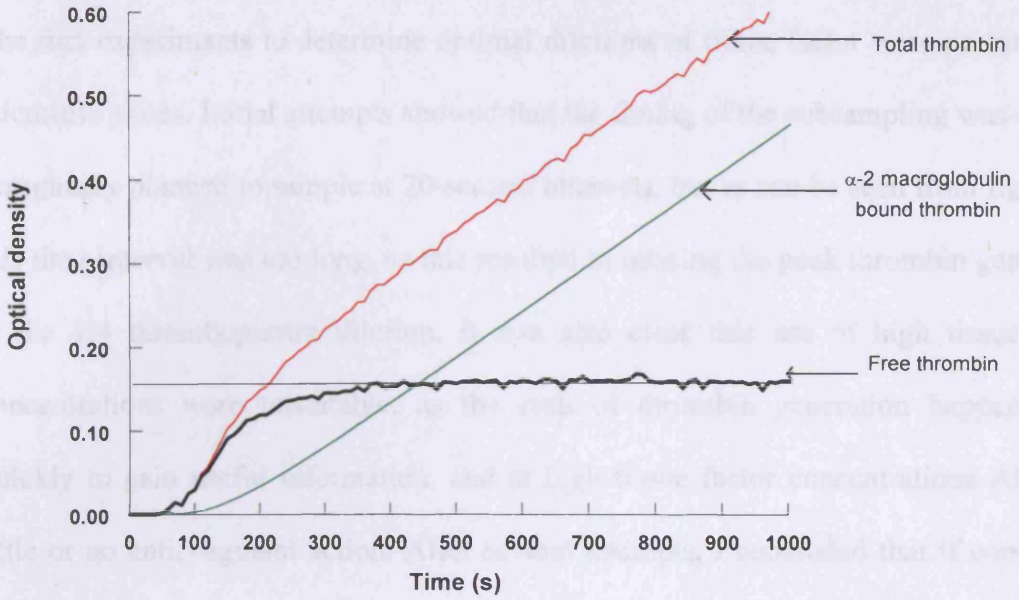

Figure 29: Graphical representation of cumulative thrombin generation. Showing total, α2 macroglobulin bound and free thrombin against time.

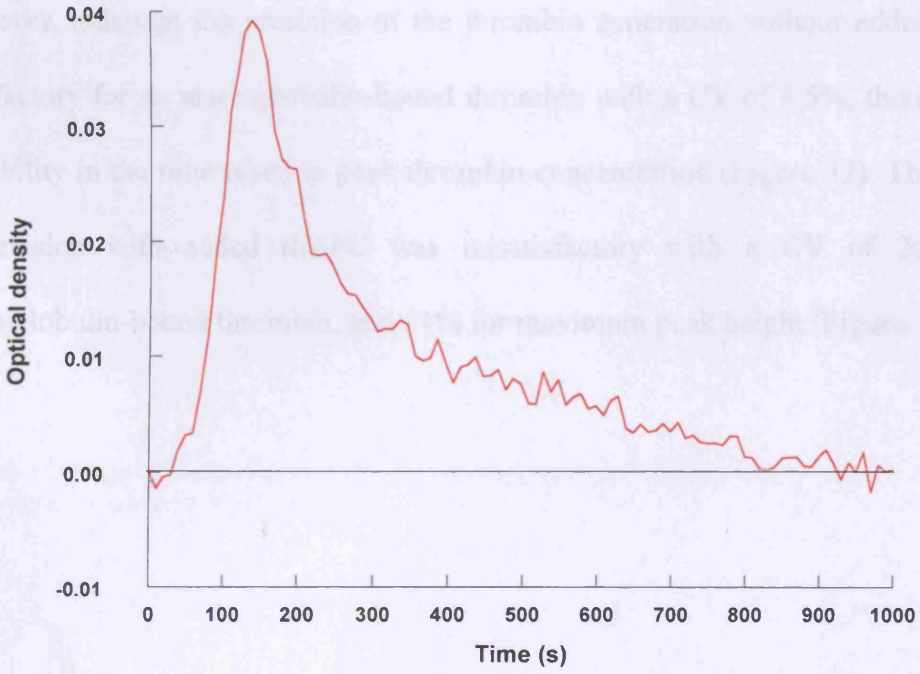

Figure 30: Graphical representation of free thrombin over time.

5.3 Results

5.3.1 Subsampling method

The first experiments to determine optimal dilutions of tissue factor were performed in microtitre plates. Initial attempts showed that the timing of the subsampling was critical. I originally planned to sample at 20-second intervals, but as can be seen from figure 31, this time interval was too long, as this resulted in missing the peak thrombin generation at the 1:4 thromboplastin dilution. It was also clear that use of high tissue factor concentrations were unsuitable, as the rush of thrombin generation happened too quickly to gain useful information, and at high tissue factor concentrations APC had little or no anticoagulant action. After several attempts, I concluded that if continuous mixing was employed, the 1:32 thromboplastin dilution gave the optimal conditions with peak thrombin generation at around 80 seconds. At this dilution, 10 nM of rhAPC reduced both peak thrombin generation and α_2 macroglobulin-bound thrombin by approximately 50%, which was ideal for my purposes (Figures 32 and 33).

However, although the precision of the thrombin generation without added APC was satisfactory for α_2 macroglobulin-bound thrombin with a CV of 4.5%, there was some variability in the time taken to peak thrombin concentration (Figure 32). The degree of imprecision with added rhAPC was unsatisfactory with a CV of 20% for α_2 macroglobulin-bound thrombin, and 21% for maximum peak height (Figure 33).

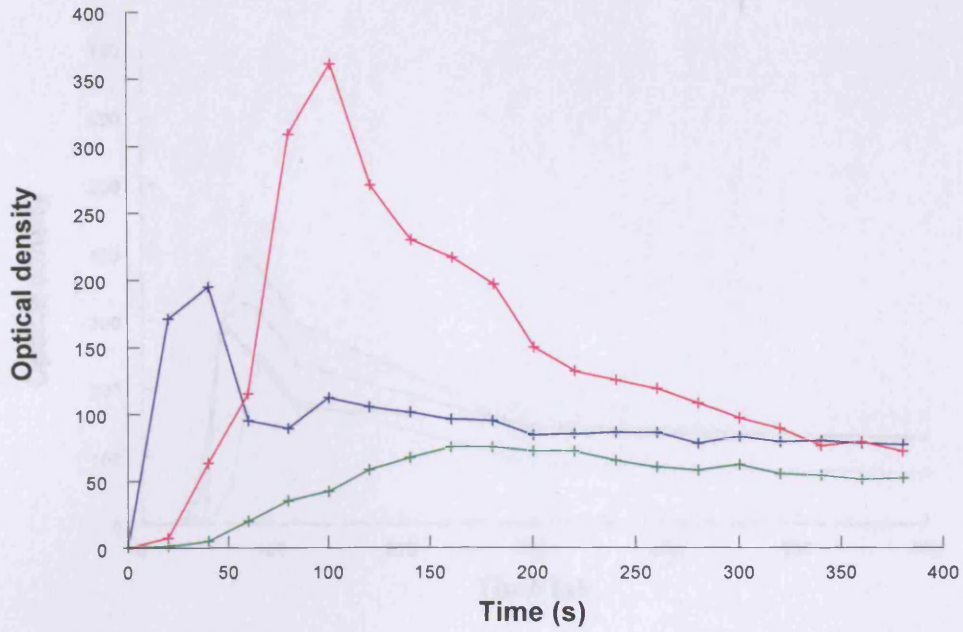

Figure 31: Titration of tissue factor to determine optimal conditions for thrombin generation.
Thromboplastin dilution 1:4 blue; 1/32 magenta; 1/128 green

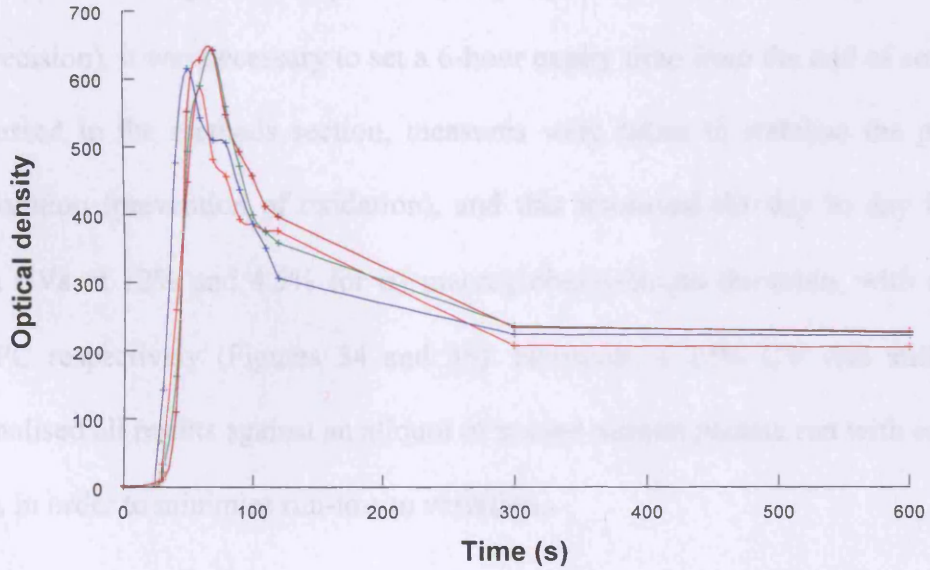

Figure 32: Initial thrombin generation curves using pooled normal plasma.
Four replicate curves are shown.

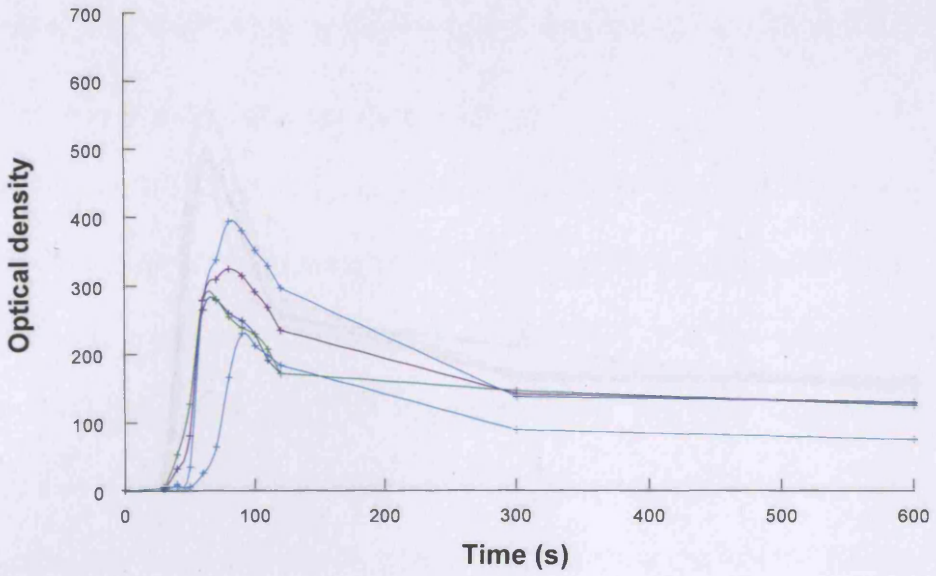

Figure 33: Initial thrombin generation curves with added APC using pooled normal plasma. Four replicate curves are shown.

As the assay was so time consuming and the phospholipid reagent frequently deteriorated during an assay run (as judged by visible opacity and increased imprecision), it was necessary to set a 6-hour expiry time from the end of sonication. As discussed in the methods section, measures were taken to stabilise the phospholipid preparation (prevention of oxidation), and this improved the day to day imprecision, with CVs of 12% and 4.5% for α_2 macroglobulin-bound thrombin, with and without rhAPC respectively (Figures 34 and 35). However, a 12% CV was still high, so I normalised all results against an aliquot of pooled normal plasma run with each batch of tests, in order to minimise run-to-run variation.

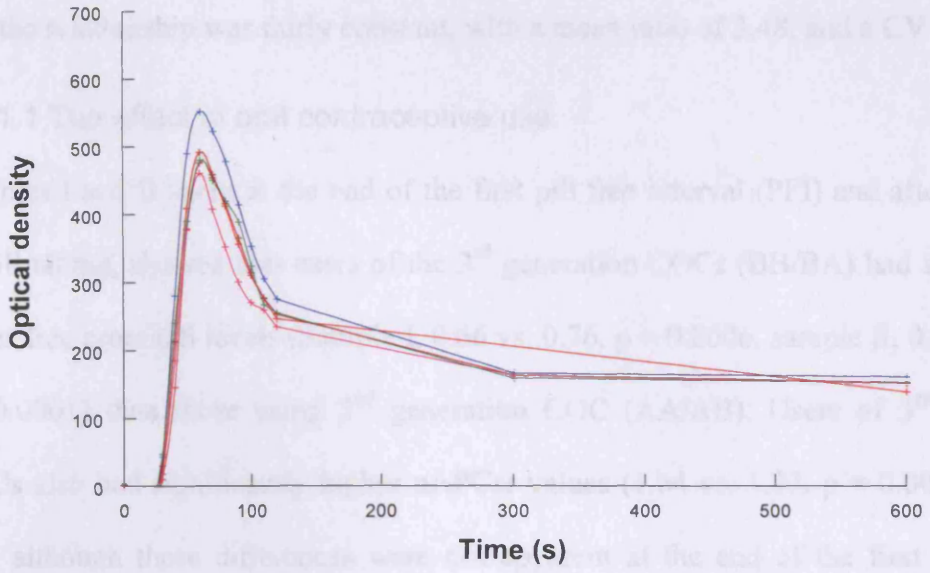

Figure 34: Thrombin generation showing improved precision after lipid stabilisation measures. (four replicate curves for pooled normal plasma are shown).

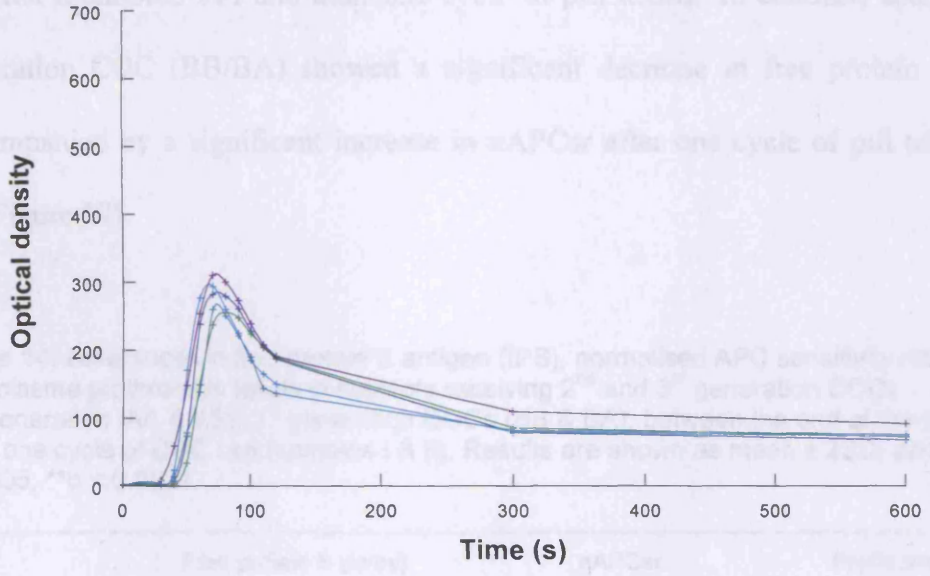

Figure 35: Thrombin generation with APC, showing improved precision after lipid stabilisation measures
(four replicate curves for pooled normal plasma are shown).

Using this subsampling method, it was not possible to calculate the area under the curve (AUC), as it uses unequal timing and this precludes the use of the available mathematical models. However, the relationship between peak height and α_2

macroglobulin-bound thrombin was studied in a pooled normal plasma. This showed that the relationship was fairly constant, with a mean ratio of 3.48, and a CV of 10.2%.

5.3.1.1 The effect of oral contraceptive use

Samples I and II taken at the end of the first pill free interval (PFI) and after one cycle of pill taking, showed that users of the 3rd generation COCs (BB/BA) had significantly lower free protein S levels (Sample I, 0.66 vs. 0.76, p = 0.0006, sample II, 0.61 vs. 0.76, p <0.0001) than those using 2nd generation COC (AA/AB). Users of 3rd generation COCs also had significantly higher nAPCsr values (1.64 vs. 1.33, p = 0.009) after the PFI, although these differences were not apparent at the end of the first COC cycle (Table 14). Users of 2nd generation COCs (AA/AB) showed a significant increase in nAPCsr but no differences in free protein S (Figure 36) between samples collected after the first monitored PFI and after one cycle of pill taking. In contrast, users of the 3rd generation COC (BB/BA) showed a significant decrease in free protein S that was accompanied by a significant increase in nAPCsr after one cycle of pill taking (Table 14, Figure 37).

Table 14: Differences in free protein S antigen (fPS), normalised APC sensitivity ratio (nAPCsr) and plasma prothrombin levels in subjects receiving 2nd and 3rd generation COCs 2nd generation (AA & AB); 3rd generation COCs (BB & BA), between the end of the first PFI and after one cycle of COC use (samples I & II). Results are shown as mean ± 2SD; paired t-test *p < 0.005, **p < 0.0001.

	Free protein S (iu/ml)		nAPCsr		Prothrombin (IU/ml)	
Sample	AA/AB	BB/BA	AA/AB	BB/BA	AA/AB	BB/BA
I (End of PFI)	0.76 ± 0.20	0.66 ± 0.18	1.33 ± 0.42	1.64 ± 0.44	1.06 ± 0.24	1.13 ± 0.36
II (After 21 pills)	0.76 ± 0.14	0.61 ± 0.18**	1.69 ± 0.58**	1.87 ± 0.60*	1.10 ± 0.34	1.20 ± 0.40*

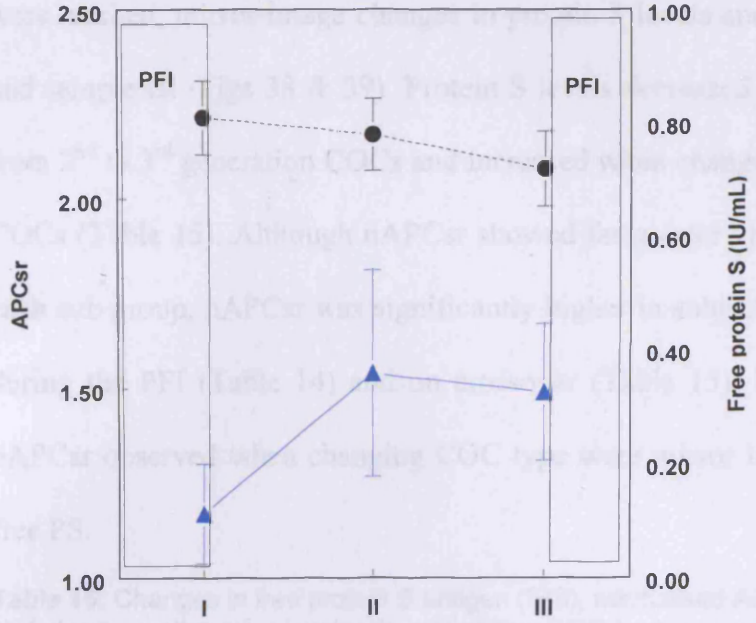

Figure 36: Resistance to APC and free protein S antigen levels for group AA
Women who remained on levonorgestrel 2nd generation (n = 6). Data is shown for free protein S antigen (λ) measured by ELISA using a specific monoclonal antibody and APC resistance expressed as the nAPCsr (σ). The error bars show the 95% confidence intervals. PFI = pill free interval.

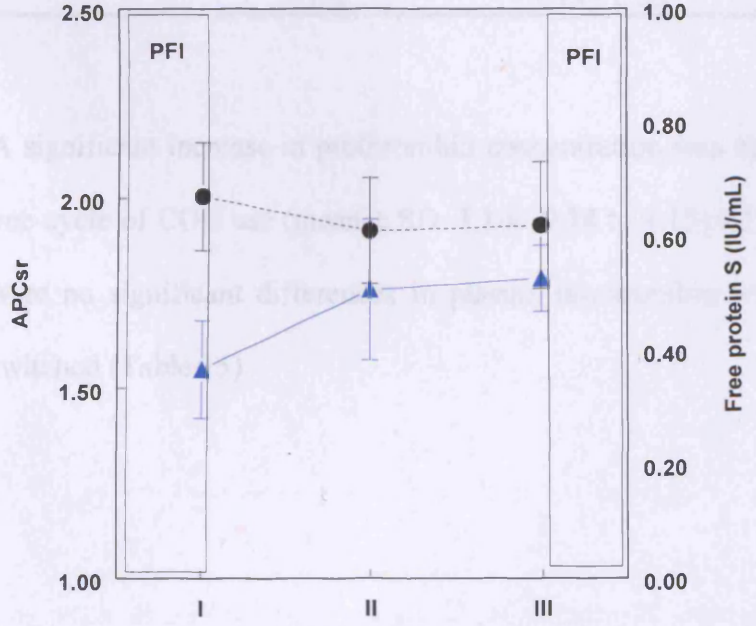

Figure 37 Resistance to APC and free protein S antigen levels for group BB
Women who remained on 3rd generation COCs (n = 7). Data is shown for free protein S antigen (λ) measured by ELISA using a specific monoclonal antibody and APC resistance expressed as the nAPCsr (σ). The error bars show the 95% confidence intervals. PFI = pill free interval.

When subjects were switched from one COC type to another (groups AB and BA), there were marked, mirror-image changes in protein S levels and nAPCsr between sample II and sample III (Figs 38 & 39). Protein S levels decreased significantly when changing from 2nd to 3rd generation COCs and increased when changing from 3rd to 2nd generation COCs (Table 15). Although nAPCsr showed far greater variability than free PS within each sub group, nAPCsr was significantly higher in subjects using 3rd generation COC during the PFI (Table 14) and on crossover (Table 15). Furthermore, the changes in nAPCsr observed when changing COC type were mirror images of those observed for free PS.

Table 15: Changes in free protein S antigen (fPS), normalised APC sensitivity ratio (nAPCsr) and plasma prothrombin levels when changing COC type.
AB = changing from 2nd to 3rd generation COC, BA = changing from 3rd to second generation COCs. Results are shown as mean ± 2SD; *p<0.05, **p<0.005, ***p<0.0001.

	Sample	II	III
		After 21 pills	After 42 pills
Free protein S (iu/ml)	AB	0.74 ± 0.14	0.59 ± 0.08***
	BA	0.60 ± 0.14	0.74 ± 0.16***
nAPCsr	AB	1.82 ± 0.36	1.92 ± 0.36*
	BA	1.97 ± 0.68	1.65 ± 0.64**
Prothrombin (IU/ml)	AB	1.09 ± 0.42	1.03 ± 0.16
	BA	1.13 ± 0.36	1.12 ± 0.36

A significant increase in prothrombin concentration was observed between the PFI and one cycle of COC use (mean ± SD, 1.10± 0.14 to 1.15±0.19 IU/ml, p = 0.01), but there were no significant differences in plasma prothrombin levels when the pill type was switched (Table 15).

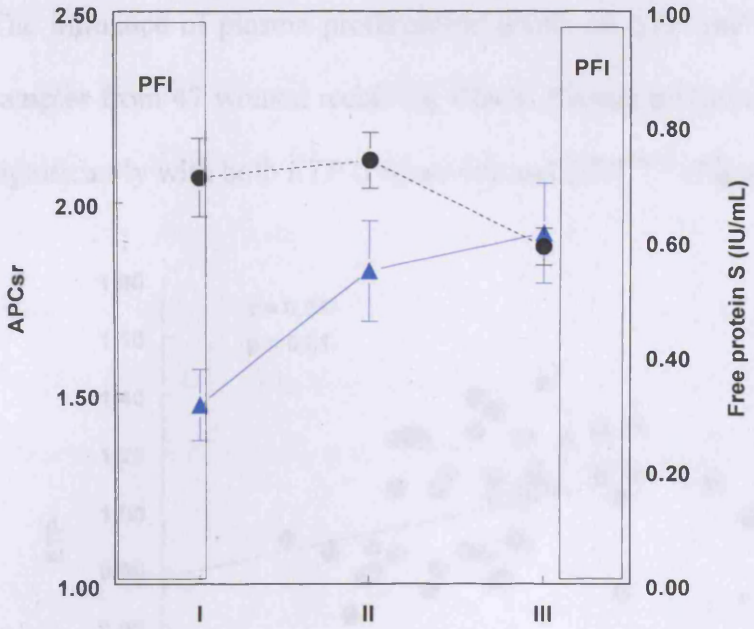

Figure 38: Group AB were long term 2nd generation COC users who switched to 3rd generation immediately after sample II (n=6).

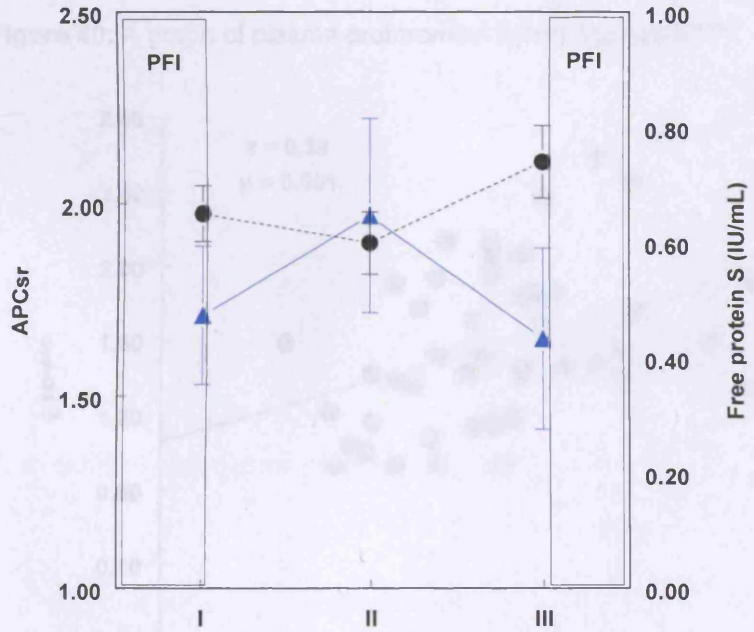

Figure 39: Group BA were long term 3rd generation COC users who switched to 2nd generation immediately after sample II (n=7).

5.3.1.2 The effect of Prothrombin

The influence of plasma prothrombin levels on *ETP* and *ETP*$^{+APC}$ was investigated in samples from 47 women receiving COCs. Plasma prothrombin concentration correlated significantly with both *ETP* (Figure 40) and *ETP*$^{+APC}$ (Figure 41).

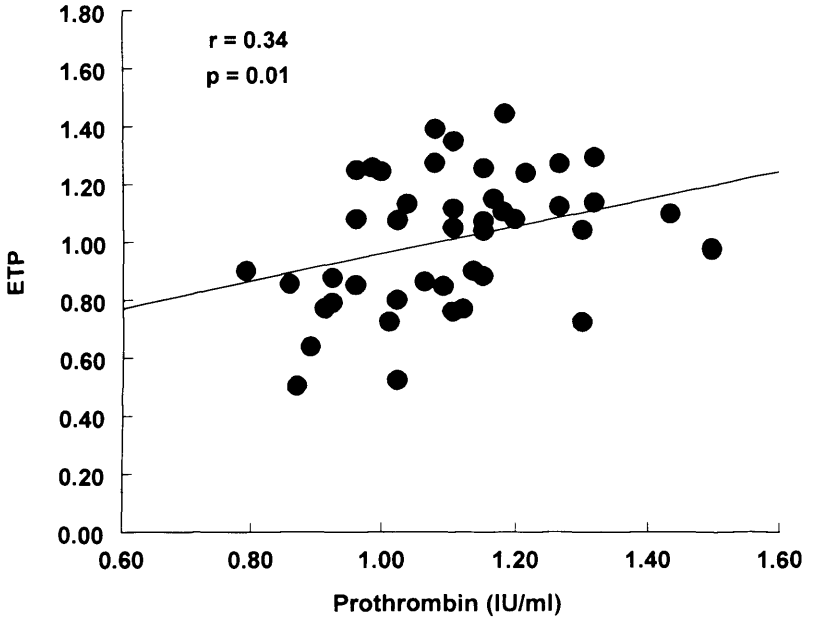

Figure 40: A graph of plasma prothrombin levels against ETP

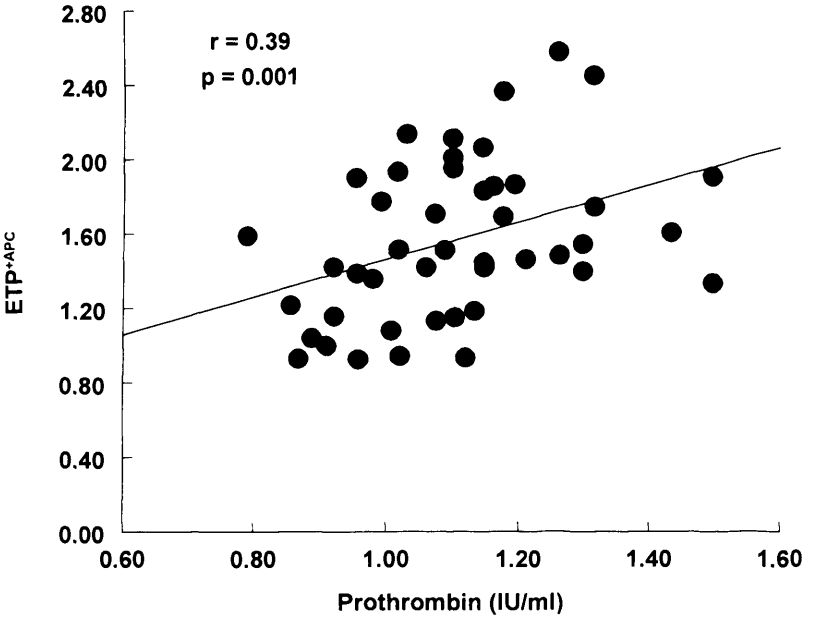

Figure 41: A graph of plasma prothrombin levels against ETP^{+APC}

5.3.1.3 The effect of plasma protein S levels

The influence of free protein S on *ETP* and *ETP*$^{+APC}$ was investigated in samples from 47 patients and controls. Free protein S had no discernable effect on *ETP* (data not shown), but showed a significant negative correlation with *ETP*$^{+APC}$ (Figure 42), despite a relatively narrow range of free protein S values. All four samples below the normal female reference range (0.58 – 1.14 IU/mL) gave ETP^{+APC} values of >1.59, and all samples with free protein S values of < 0.70 IU/mL gave ETP^{+APC} values of >1.20 suggesting that this method is sensitive to reduced protein S levels.

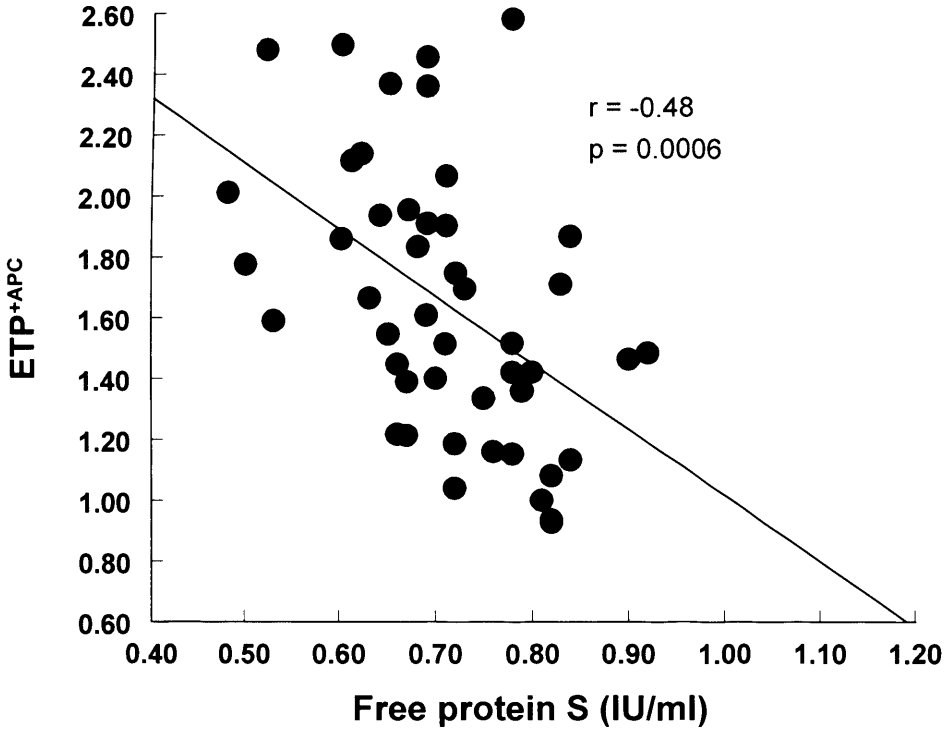

Figure 42: A graph of free protein S against *ETP*$^{+APC}$

5.3.1.4 The effect of antiphospholipid antibodies

Having obtained a degree of standardisation using the subsampling method, I studied thrombin generation in sixteen patients (not receiving anticoagulants) with APS (Table 16). These patients had a broad range of clinical and laboratory findings, which I felt,

were representative of aPA positive patients. Ten of the sixteen patients had high nAPCsr as judged by their α_2 macroglobulin-bound thrombin levels.

Table 16: Characteristics of aPA positive patients studied

VTE = venous thromboembolism, IUFD = intrauterine fetal death, CI = cerebral ischaemia, FH = family history. aCL = anticardiolipin, β2GPI = anti-β2 glycoprotein-I.

Patient	Clinical details	dRVVT	IgG aCL	IgM aCL	β_2GPI
1	VTE familial APS	1.37	>84	6.0	29.9
2	SLE stroke	2.14	89.6	5.2	56.8
3	IUFD + CI	1.45	80.5	7.2	19.2
4	SLE VTE	1.15	67.5	61.1	5.0
5	VTE	0.98	45.7	3.4	44.2
6	RM migraine	1.08	22.3	0	0.6
7	VTE	1.21	18.5	0	10.7
8	IUFD	1.15	7.2	0	34.2
9	TIA+ RM	0.98	4.7	0.2	5.3
10	Livedo and VTE	0.98	4.7	0.2	5.3
11	CI	1.62	0.9	0.3	1.6
12	FH VTE, asymptomatic	1.23	0.3	0	nd
13	APA	1.08	18.7	8.2	2.9
14	TIA ITP	1.66	4	3.0	5.0
15	DVT	1.00	17.6	0.6	3.3
16	CVA	1.02	9.5	0.0	0.3

Table 17: Thrombin generation in patients with aPA studied
Normal values are indicated by black type, abnormally low values in blue and raised values in red. Reference ranges from the automated method: nAPCsr 0.57 – 1.23, ETP 0.73 – 1.06 and ETP+APC 0.42 – 1.07

Patient	Clinical details	nAPCsr	ETP	ETP^{+APC}
1	VTE familial APS	2.57	0.33	0.85
2	SLE stroke	2.25	0.22	0.49
3	IUFD + CI	1.16	0.61	0.73
4	SLE VTE	0.86	1.11	0.96
5	VTE	1.48	1.26	1.86
6	RM migraine	1.00	0.63	0.62
7	VTE	1.45	0.91	1.31
8	IUFD	0.75	0.71	0.54
9	TIA+ RM	1.36	0.88	1.19
10	Livedo and VTE	1.25	1.23	1.56
11	CI	1.48	1.12	1.70
12	FH VTE, asymptomatic	0.73	1.30	0.97
13	SLE +CI	2.51	0.66	1.65
14	TIA ITP	1.70	0.59	1.00
15	DVT	1.61	0.66	1.07
16	CVA	0.46	1.50	0.69

This preliminary data suggested, the majority of patients with aPA had an abnormal nAPCsr ratio. I studied the raw data from these patients and found several different patterns of thrombin generation that the nAPCsr alone could not show. If the amount of thrombin generated with and with out APC were expressed relative to that of pooled normal plasma, i.e., ETP^{+APC} and ETP, it became apparent that some plasmas with raised nAPCsr had low ETP and ETP^{+APC}, some has raised ETP and ETP^{+APC}, while others had normal ETP and raised ETP^{+APC} (Table 17).

When I examined the actual thrombin generation curves themselves, the picture became more complicated still. Figure 43 shows the curves from patient 1. The time of peak thrombin formation was similar for both the patient and normal plasma, with the addition of APC giving a slightly longer time. It was clear that the amount of thrombin produced by the patient's plasma was significantly less than that of the normal plasma in the absence of APC. However, broadly similar amounts of thrombin were generated in both plasmas when APC was added. Although there was a poor anticoagulant response to APC, as shown by the nAPCsr, the amount of thrombin generated in the presence of APC was normal.

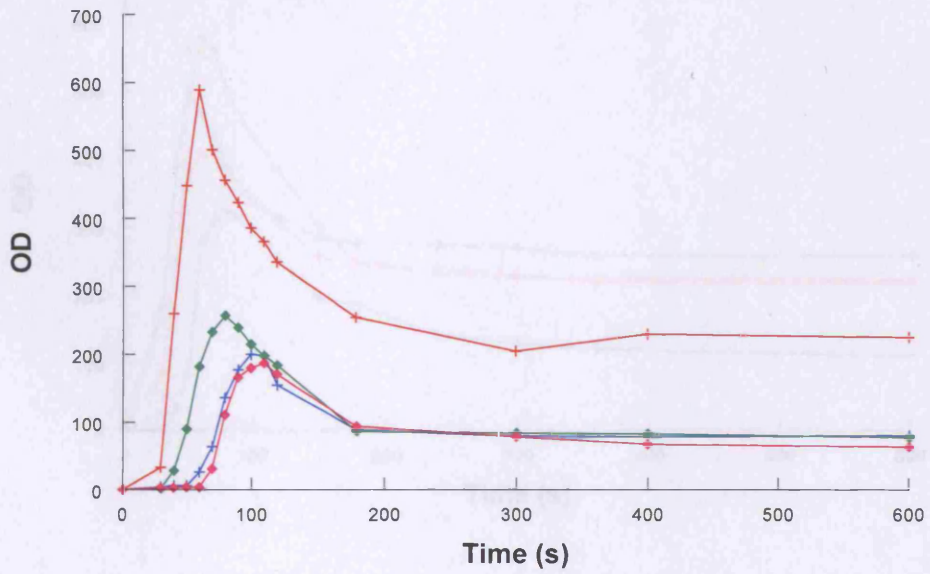

Figure 43: Thrombin generation curves from patient 1.
Pooled normal plasma without APC is shown in red +
Pooled normal plasma with APC is shown in blue +
Patient's plasma without APC is shown in green ♦.
Patient's plasma with APC is shown in magenta ♦.

Patient 10 had a raised nAPCsr, raised ETP and ETP^{+APC}. The thrombin generation curves show a faster rate of thrombin generation, as judged by a reduced lag phase prior to thrombin generation and a shorter time to peak thrombin generation (Figure 44). In the absence of APC, peak thrombin generation in the patient's plasma is less than that of the normal plasma, although, the α_2 macroglobulin-bound thrombin are the opposite. When APC is added, both the peak and α_2 macroglobulin-bound thrombin levels were much higher than that of the normal plasma. Indeed the patient plasma produced levels of thrombin in the presence of APC that were similar to those seen in normal plasma without APC, i.e. a considerable excess of thrombin was produced.

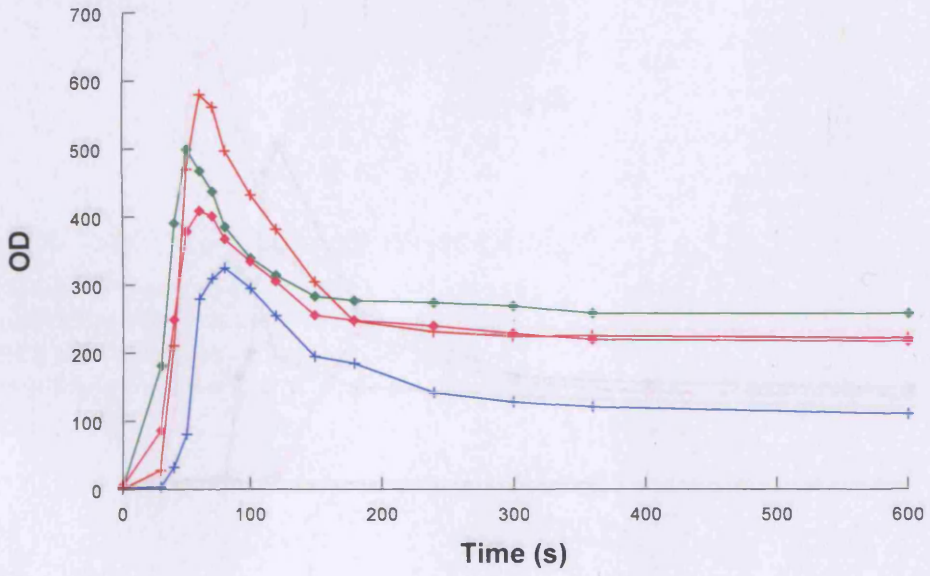

Figure 44: Thrombin generation curves from patient 10.

Pooled normal plasma without APC is shown in red +
Pooled normal plasma with APC is shown in blue +
Patient's plasma without APC is shown in green ♦.
Patient's plasma with APC is shown in magenta ♦.

Patient 14 had a strong lupus anticoagulant, and this was reflected by a long lag phase before thrombin generation and peak thrombin generation at >120 seconds (Figure 45). As the subsampling intervals were longer after this time, it appears that I missed the peak thrombin generation with APC in this plasma. However, the final α_2 macroglobulin-bound thrombin levels with and without APC are almost identical, suggesting that the peak heights would have been similar, i.e., an almost complete resistance to APC. In order to capture the peak thrombin generation times in patients such as this, regular subsampling would have to be performed over a much wider time frame.

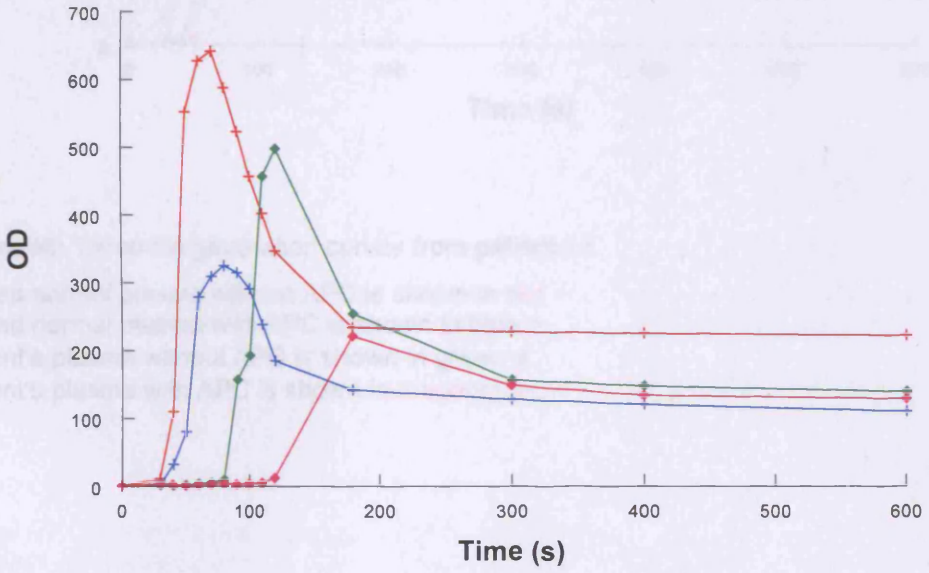

Figure 45: Thrombin generation curves from patient 14.

Pooled normal plasma without APC is shown in red +
Pooled normal plasma with APC is shown in blue +
Patient's plasma without APC is shown in green ♦.
Patient's plasma with APC is shown in magenta ♦.

Plasma from patient 13 demonstrated a slight increase in lag time and time of peak thrombin generation (Figure 46). The patient's plasma generated less thrombin that the normal plasma in the absence of APC, but again, the patient's plasma demonstrated a poor anticoagulant response to APC.

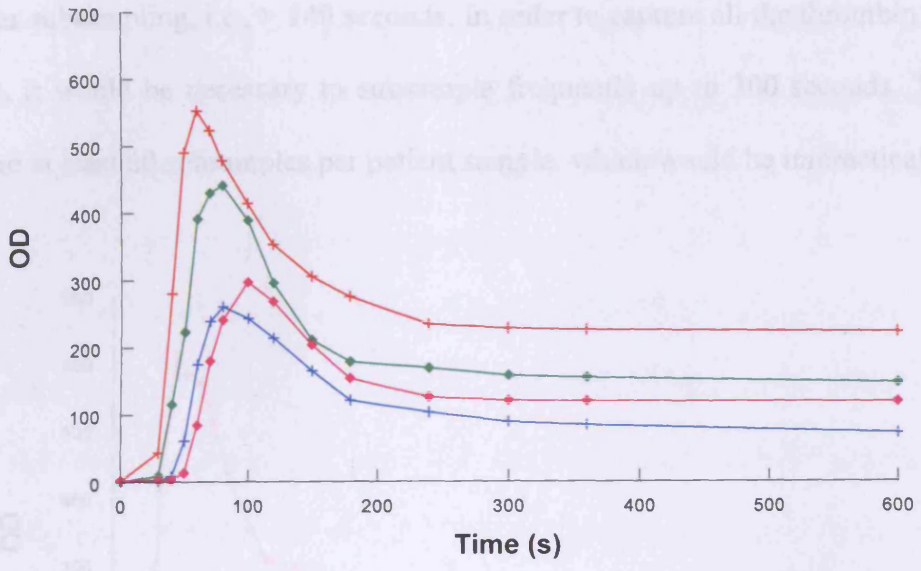

Figure 46: Thrombin generation curves from patient 13.

Pooled normal plasma without APC is shown in red +
Pooled normal plasma with APC is shown in blue +
Patient's plasma without APC is shown in green ♦.
Patient's plasma with APC is shown in magenta ♦.

Plasma from several patients showed reduced thrombin generation and increased lag phase and time to peak thrombin generation, yet a very poor response to APC. Figure 47, which shows the thrombin generation curves from patient 2, was typical of this type of response. It is clear that, once again, that the thrombin generation peak for the patient's plasma was not detected, as this would have occurred after the period of regular subsampling, i.e., > 140 seconds. In order to capture all the thrombin generation peaks, it would be necessary to subsample frequently up to 300 seconds. This would require at least 60 subsamples per patient sample, which would be impractical.

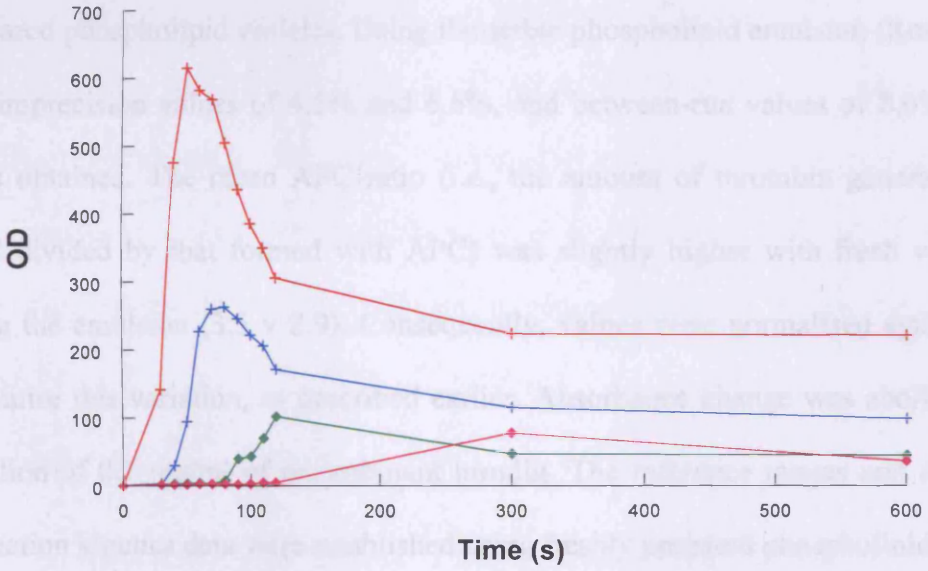

Figure 47: Thrombin generation curves from patient 1.
Pooled normal plasma without APC is shown in red
Pooled normal plasma with APC is shown in blue
Patient's plasma without APC is shown in green.
Patient's plasma with APC is shown in magenta.

The subsampling technique proved to be very useful in showing the heterogeneity of thrombin generation and response to APC in patients with aPA, but the method was exceptionally labour intensive and did not cover all relevant time points. It was clear that a more practical method would be required if larger number of samples were to be studied.

5.3.2 Automated thrombin generation method

By using a tissue factor concentration of approximately 7pM (Innovin diluted at 1:600) as judged by a tissue factor ELISA, it was possible to achieve an APC ratio (thrombin generation without APC: thrombin generation with APC approximately 3:1). Tissue factor ELISA remain poorly standardised, as there is no international standard for the determination of relative potencies, and assays are frequently non-parallel. Consequently, I have used both the dilution and assayed tissue factor concentrations throughout this thesis. Within assay imprecision for *ETP* and ETP^{+APC} was 4.2% and 10.6% respectively, with between run imprecision of 9.9% and 15.4% using freshly prepared phospholipid vesicles. Using the stable phospholipid emulsion (Rossix), within run imprecision values of 4.5% and 6.5%, and between-run values of 8.6% and 7.9% were obtained. The mean APC ratio (i.e., the amount of thrombin generated without APC divided by that formed with APC) was slightly higher with fresh vesicles than using the emulsion (3.5 v 2.9). Consequently, values were normalised against PNP to minimise this variation, as described earlier. Absorbance change was abolished by the addition of 0.1 mg/ml of recombinant hirudin. The reference ranges and all thrombin formation kinetics data were established using freshly prepared phospholipid vesicles.

Normal reference ranges were established in 30 healthy normals (20 females, 10 males; mean age 34 years) not using drugs known to affect the coagulation system. There was no difference in median *ETP* between male and females, but women gave significantly higher ETP^{+APC} values than men (Table 18). The mean time of peak thrombin formation was 98 seconds (standard deviation 14 seconds) without APC and 113 seconds (standard deviation 21 seconds) with APC.

Table 18: Reference ranges for *ETP* and ETP^{+APC}: Median (2.5^{th} - 97.5^{th} percentiles)

	Male (n=10)	Female (n=20)	All (n=30)
ETP	0.90 (0.73-1.05)	0.93 (0.77-1.06)	0.93 (0.73-1.06)
ETP^{+APC}	0.63** (0.41-0.95)	0.89 **(0.72-1.07)	0.83 (0.42-1.07)
nAPCsr	0.74 (0.56-0.93)	1.01 (0.80-1.23)	0.91 (0.57-1.23)

**P = 0.0001

As would be expected, factor V Leiden caused a marked increase in ETP^{+APC} in the seven heterozygous patients studied (median 1.84 [IQR 1.77-2.32, P < 0.0001]). A small but significant increase in *ETP* was also observed (median 1.07; IQR 0.8-1.28).

Although women receiving the combined oral contraceptive pill (COC) were excluded from the normal reference range, eight additional women receiving COCs were tested. As I have demonstrated using the subsampling method, COC use is associated with APC resistance in thrombin generation assays and both the *ETP* and ETP^{+APC} values were significantly higher than female reference ranges (*ETP* median 1.03 [95% range0.86 - 1.23]; ETP^{+APC} 1.26 [1.07 - 1.68]).

5.3.3. The effect of plasma factor VIII levels

In developing the automated method, I had reduced the tissue factor concentration with the aim of making the method sensitive to factor VIII. In order to study the effect of factor VIII concentration on *ETP* and ETP^{+APC}, varying quantities of factor VIII concentrate (Alphanate®, Grifols, Cambridge, UK) was added to plasma from a patient with severe haemophilia A. Increasing factor VIII concentration caused a dose dependent increase in both *ETP* and ETP^{+APC} with near normal levels observed at a factor VIII concentration of 0.10 IU/ml (Figure 48).

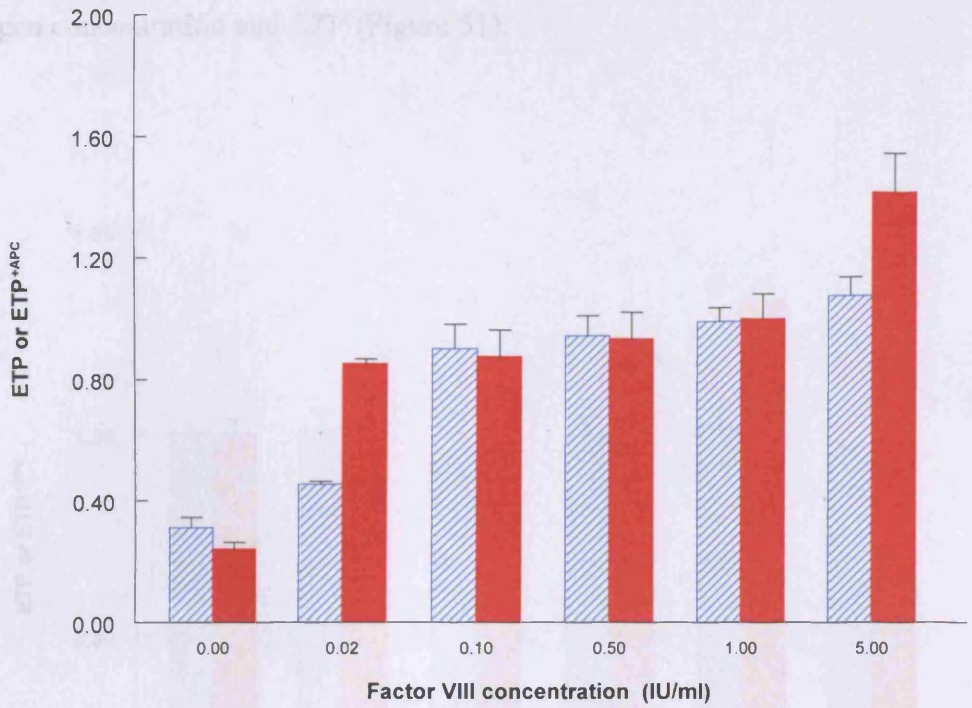

Figure 48: The effect of plasma factor VIII concentration on thrombin generation. Purified factor VIII concentrate was added to plasma from a severe haemophiliac. *ETP* is represented by the shaded blue bars and ETP^{+APC} is represented by solid red bars.

5.3.4. The effect of plasma tissue factor pathway inhibitor levels

In order to investigate the influence of tissue factor pathway inhibitor (TFPI) activity on *ETP* and ETP^{+APC}, a polyclonal antibody (rabbit anti-human TFPI IgG, American Diagnostica Inc.) known to block TFPI function, was added to normal plasma to give concentrations of 12.5 – 200 ng/mL i.e., a maximum of 0.2 mL of antibody at 1.25 g/L were added to 1ml of plasma. Although a buffer control was used to control for the effect of dilution, an irrelevant antibody control was not used. Thrombin generation was performed with and without rhAPC on the ACL9000 using the continuous measurement method. The progressive blocking of TFPI function caused a dose dependent increase in ETP^{+APC} of up to 50% in normal plasma and a modest decrease in *ETP* (Figure 49). Furthermore, when TFPI antigen and thrombin generation were studied in 32 plasma samples from a range of patients (aPA positive and negative, but no factor V Leiden) and controls, a significant negative correlation was observed between TFPI antigen and

ETP^{+APC} (Figure 50; r = -0.48, P = 0.001), but no correlation was found between TFPI antigen concentration and *ETP* (Figure 51).

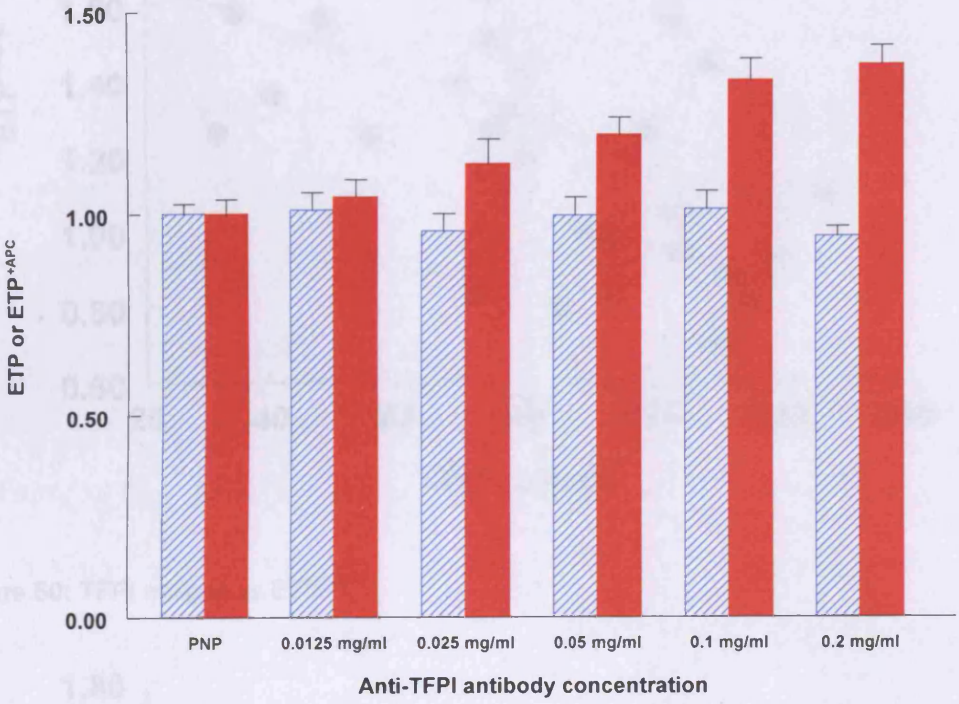

Figure 49:. *ETP* is represented by the shaded blue bars and ETP^{+APC} is represented by solid red bars

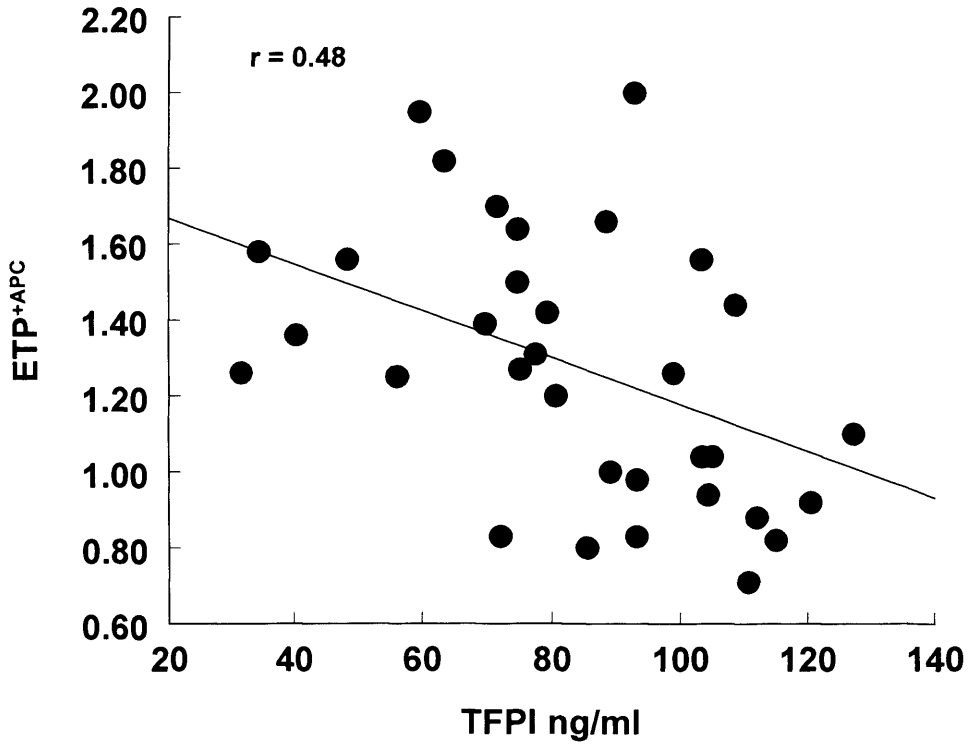

Figure 50: TFPI antigen vs ETP^{+APC}

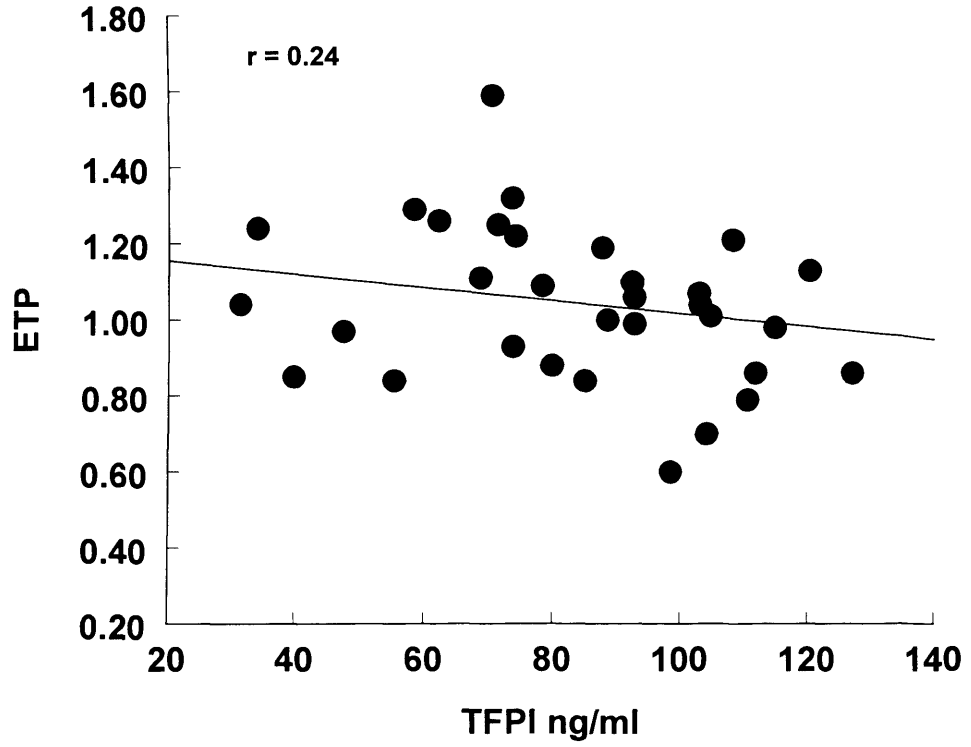

Figure 51: TFPI antigen vs. ETP

5.4 Discussion

Although the results from my RVV clotting test were encouraging, there is no routine test that quantitatively measures the thrombin forming capacity of a plasma sample. In order to understand the mechanisms by which aPA caused resistance to APC, it was necessary to develop a method to assess thrombin generation in the presence of APC. Initially I concentrated on developing a manual sub-sampling technique. APC resistance was measured as a sensitivity ratio (nAPCsr) in an amidolytic assay of tissue factor initiated thrombin generation, with and without exogenous rhAPC.

Tissue factor ELISA remain poorly standardised, as there is no international standard for the determination of relative potencies, and assays are frequently non-parallel. Furthermore, the manufacturers of thromboplastin reagents containing recombinant human tissue factor do not routinely provide the tissue factor concentrations for their reagents. Consequently, while I have attempted to estimate the tissue factor concentration wherever possible, I believe that the dilution may be more reliable and I have used both throughout this thesis.

Initial experiments using Innovin diluted at approximately 1/8 resulted in consistent thrombin generation, but no detectable APC activity at 5nM. Increasing the APC concentration did not seem to be a reasonable option as this would require plasma APC concentrations far in excess of those seen in vivo. I inferred that the absence of detectable APC activity was most probably due to the rapid accumulation of FVa having a swamping effect on the APC as previously described (Curvers et al. 2002a), and I subsequently improved the sensitivity by reducing the tissue factor concentration. It was found that using thromboplastin within 8 hours of reconstitution, and the use of freshly prepared buffers improved the precision of the method. I identified the phospholipid preparation as the other important source of variability and overcame this

by storing multiple aliquots of a single preparation under nitrogen, at -80°C, thus preventing oxidation.

5.4.1 Oral contraceptive use

Thrombin generation assays are notoriously difficult to standardise, due to methodological variation and the technical difficulty. My aims were twofold: I wanted to evaluate the utility of the ETP for the detection of small changes in haemostatic systems; I also wanted to corroborate the findings of increased APC resistance associated with 3rd generation COC.

There are several potential explanations for the reported excess risk of venous thrombosis associated with 3rd generation COCs compared to 2nd generation COCs. Rosing *et al* (1997) reported that users of the 3rd generation COC demonstrated an acquired increase in resistance to APC, measured in endogenous thrombin potential (ETP) assays, which resulted in excess thrombin generation, and that the ETP based APC resistance is a risk factor for venous thromboembolism (Tans et al 2003).

A number of previous studies have shown that protein S is decreased in women receiving COC pills (Archer, Mammen, & Grubb 1999; Granata et al. 1991; Winkler et al. 1999)It is known that Ethinylestradiol alone, may cause decreased protein S, and this may take 4 weeks to reverse (van Ommen et al. 1999). In sample I of this study, protein S levels were lower in users of 3rd generation COCs than 2nd generation COCs. However, although users of both types showed a significant increase in nAPCsr and plasma prothrombin concentration, only women receiving 3rd generation COCs showed a concomitant decrease in protein S levels. Although I did not find a significant relationship between plasma prothrombin concentration and nAPCsr, prothrombin concentration did correlate, albeit weakly, with ETP and ETP^{+APC}, so it is probable that the differences in nAPCsr between pill types and between samples I and II were due in

part to the increase in prothrombin levels (Curvers et al. 2002b). Switching from 2nd to 3rd generation COCs caused a significant decrease in protein S antigen, mirrored when switching COCs in reverse fashion. Tans et al (2000) have shown a similar decrease in protein S in association with DSG COC. While inherited protein S deficiency is associated with an increased risk of thrombosis, and family studies (Simioni et al. 1999) have shown a high relative risk of VTE in congenital protein S or C deficiency compared to non-carriers. However, the exact relationship between protein S deficiency and thrombosis has recently been questioned (Liberti et al 1999).I have shown a strong association between protein S levels and nAPCsr.

This study independently confirms Rosing's observations (1997), that nAPCsr is higher in women receiving DSG than LNG containing COCs. It is probable that both increased plasma prothrombin and reduced protein S levels contribute to the loss of APC co-factor activity and hence the relatively increased nAPCsr results linked with 3rd generation COCs. Tissue factor pathway inhibitor (TFPI) affects the initiation phase of coagulation and, as I have shown, may influence ETP APC sensitivity assays. It was not possible to measure TFPI levels in these samples (due to insufficient stored plasma) but as reduced TFPI levels have been reported in COC users (Dahm et al. 2006), this may also have influenced the results. A recent study (Kemmeren et al. 2004) suggested that the differential effects of 2nd and 3rd generation COCs on the protein C pathway are due in part to the greater protective effect of levonorgestrel on the estrogen-mediated effects on protein S levels. This, in association with APCR, may be implicated in the pathophysiology of thrombosis in COC users. It is likely that COC users whose nAPCsr are in the highest percentiles may be at greatest risk, but a further triggering factor may be necessary before actual thrombosis occurs.

5.4.2 Antiphospholipid antibodies

These initial results suggested that the subsampling method was a sensitive indicator for abnormalities in the protein C anticoagulant pathway. I was keen to find out whether I could use the same technique to study the procoagulant effects of antiphospholipid antibodies. I studied plasma from sixteen patients with aPA, and found abnormalities in the thrombin generation curves in twelve. While increased nAPCsr were found in eleven patients, the underlying abnormalities were very heterogeneous. Thrombin generation, in the absence of APC, was reduced, normal or increased, with normal times for peak thrombin generation, accelerated thrombin generation and delayed thrombin generation all observed. In most cases thrombin generation in the presence of APC was normal or increased. In some cases, a prolonged lag time to the start of thrombin generation and delayed peak thrombin generation, analogous to the lupus anticoagulant effect were observed in association with increased thrombin generation in the presence of APC. If these results were representative of APS patients, they could go a long way towards explaining the prothrombotic phenotype observed in APS. Although antithrombin is the principal inhibitor of free thrombin, the protein C pathway is the major feedback mechanism to control the amount of thrombin generated in vivo. The protein C pathway also plays a major role in localising thrombin generation to the site of injury as it has been reported that APC does not regulate thrombin production on the platelet surface (Camire et al. 1998; Taube et al 1999) but acts on the vascular endothelium(Oliver et al 2002), although this has been disputed (Briede et al. 2001). Consequently, although excessive thrombin generation *per se* is strongly associated with hypercoagulable states, the amount of thrombin formed in the presence of APC is probably more significant than the total amount of thrombin generated in its absence, when determining thrombotic risk. This is particularly true of the aPA, as it has been

reported that overall thrombin generation is reduced in the majority of cases, which would be expected to cause a bleeding disorder rather than thrombosis.

However, there were several drawbacks associated with using this subsampling method to study aPA. The method was labour intensive, involving in excess of 60 pipetting steps per sample tested. No more than four patient samples per day could be tested. Even when taking 36 subsamples from each sample, it was possible to miss the peak thrombin generation, either due to accelerated thrombin generation with a short peak, or delayed thrombin generation giving a peak after the end of frequent sampling. It was simply not practical to subsample every 10 seconds for 3-4 minutes for each limb of the test. The subsampling requires a high degree of dexterity and accurate timing is paramount. Not surprisingly, it was not always possible to achieve the degree of precision required and some samples are wasted as a result.

5.4.3 Continuous automated monitoring of thrombin generation

While the subsampling thrombin generation method yielded some very interesting results, it was clear that I would have to develop a less labour intensive method to measure thrombin generation and sensitivity to APC. As I did not have access to specialised equipment at the time, a method which could be performed using a standard laboratory coagulometer was highly desirable. In order to achieve this, several problems had to be addressed. The short time interval between measurements resulted in a 'noisy' signal, which required a moving average logarithm to smooth the signal and allow the results to be recorded and displayed graphically.

The selection of tissue factor concentration was critical, as too high a concentration would lead to the rapid formation of factor Va, thus swamping the APC, while at concentrations of <5pM, interference from *in vitro* contact activation can be a major factor (Luddington & Baglin 2004), requiring the addition of corn trypsin inhibitor to blood collection tubes for its abolition. Furthermore, at low tissue factor concentrations,

the TFPI dependent action of protein S far exceeds its APC cofactor activity (Hackeng et al. 2006). I found that at tissue factor concentrations in excess of 8pM, the speed of the reaction swamped the APC, so that APC concentrations in excess of 20nM were required. By using a tissue factor concentration of approximately 7pM (Innovin diluted at 1:600) it was possible to achieve a useful APC ratio (thrombin generation without APC: thrombin generation with APC approximately 2.5:1). Increasing the concentration of APC is known to increase the APC ratio (Nicolaes et al 1997), but in my assay this was at the expense of increased imprecision in the ETP^{+APC} measurement. There was still some between assay variation, and normalisation against pooled normal plasma (PNP) was used as previously suggested (Curvers et al 2002b; Nicolaes et al 1997).

Traditionally, ETP based APC sensitivity tests have been reported as a normalised ratio of the ETP value with and without APC relative to PNP. A major drawback of this approach is that the ratio is not quantitative, as it does not take into account the baseline level of thrombin generation. This makes the normalised ratio relatively insensitive to factor VIII concentration (de Visser et al 2005). By measuring the amount of thrombin produced relative to that of PNP, and reducing the tissue factor concentration, I have been able to make the assay sensitive to increased factor VIII concentration. Rosing *et al* (2004) has suggested that using this approach to detect the failure of the protein C system to down regulate excessive thrombin generation rather than using a simple ratio, may be more representative of physiological processes.

In a system such as this, it is necessary to provide a phospholipid surface for both thrombin generation and for the APC complex to form. The source of phospholipids used for the assay proved to be a difficult decision. Initially I used freshly prepared phospholipid vesicles. This produced a well-defined thrombin generation peak, but the preparation was time consuming, and their stability was limited to 6 hours. Using a stable phospholipid emulsion (kindly provided by Steffen Rosen at Rossix) significantly

reduced the time required to perform the assay and gave very similar results in terms of the quantities of thrombin generated and, although the APC ratio was slightly lower, the precision of the method improved. However, the signal was 'noisier' than that obtained using the freshly prepared vesicles, so that it was frequently difficult to obtain a well-defined thrombin peak or determine the lag phase prior to the thrombin generation burst. The method by which the ACL7000 mixes the plasma and reagents in the test rotors creates turbulence, which can result in a noisy signal, and this is exacerbated by turbidity in the reagents or plasma. As I was primarily interested in the total amount of thrombin generated, rather than the kinetics, this did not present a significant problem. However, if this method was to be used to study the kinetics of thrombin generation, freshly produced vesicles, or some way of reducing turbulence during measurement would be required. Due to this turbulence and the relatively high concentration of tissue factor used in this method, little useful information about the lag time before thrombin formation could be obtained.

A limitation of any chromogenic method for measuring thrombin generation is that the plasma must be defibrinated prior to use. Fibrin does not readily allow the passage of light and so the large change in optical density at the point of coagulation masks the smaller changes in OD caused by the cleavage of the substrate. Clearly this precludes the use of platelet rich plasma (PRP). While the use of PRP undoubtedly provides a great deal of information about thrombin generation on the platelet surface, it is doubtful that it tells us a great deal about the anticoagulant pathways. It is now generally accepted that the site of thrombin generation is different from the site of APC dependent degradation of factor Va and VIIIa (Hoffman et al 2001). Oliver et al (2002) showed that APC activity was considerably more potent on phospholipid surfaces and endothelial cells than platelets. For this reason, when APC resistance is studied in PRP

using thrombin generation, relatively high concentrations of APC are required even at low tissue factor concentrations (Regnault et al. 2003).

My assay is sensitive to the factor V Leiden mutation, COC use, increased levels of prothrombin, and reduced levels of protein S and TFPI as been previously described for the ETP-based normalised APC ratio (*nAPCsr*) (de Visser et al 2005). However, as I used a quantitative approach to measure ETP^{+APC}, my assay was also sensitive to factor VIII concentration. It has been suggested that raised thrombin generation alone is predictive of deep vein thrombosis (Brummel-Ziedins et al. 2005), although the reported odds ratio was relatively low (OR 1.6). Tans et al (2003) reported that the *nAPCsr* was a more useful test in determining thrombotic risk (odds ratio of 2.4 for women and 7.5 for men). As ETP^{+APC} is sensitive to additional risk factors, it is interesting to speculate whether this measurement could be more useful than the *nAPCsr* in this context.

My preliminary data suggested that, in the aPA positive patients studied, thrombin generation was abnormal, with a variable anticoagulant response to APC. It has also been possible to establish a possible mode of action for lupus anticoagulants, which explains both the observed prolonged *in vitro* clotting time and the paradoxical increase in markers of in vivo thrombin generation and thrombotic risk, i.e., delayed onset of thrombin generation, which is analogous to a prolonged clotting time, with a failure of APC to inhibit thrombin generation. In developing and characterising a more rapid automated method for ETP-based sensitivity to APC, I paved the way for a more in depth study of APC resistance associated with the antiphospholipid syndrome.

6. A Study of the Protein C Pathway in Patients with Antiphospholipid Antibodies

6.1 Introduction

There are conflicting reports about aPA and thrombin generation. It has been reported that aPA increase *in vitro* thrombin generation (Adams et al 2004; Lean et al. 2006; Liestol et al 2007; Rand et al 1999), prolong the time to peak thrombin generation and reduce peak thrombin generation (Regnault et al 2003), or inhibit thrombin generation (Hanly et al 2000; Sheng et al 2001a). Several authors have implicated aPA in the inhibition of APC (Aznar et al 1997; Gennari et al. 2002), with IgG with LA activity (Borrell et al. 1992; Nojima et al. 2002; Regnault et al 2003) and anti β_2GPI reported to be the antibodies responsible (Galli et al 1998; Gennari et al. 2003; Martinuzzo et al 1996; Mercier et al 1998; Viveros et al 2005). It has been reported that β_2GPI is necessary for the inhibition of protein C activity by IgM human monoclonal anticardiolipin antibodies (Ieko et al 1999) and that this may be achieved by increased binding of β_2GPI to phospholipid surfaces facilitated by the dimerisation of β_2GPI by aPA molecules (Takeya et al 1997). To date, the only published studies of aPA associated APC resistance, that used a thrombin generation-based assay (Lecompte et al. 2007; Regnault et al 2003) reported that IgG from 7/8 patients with LA inhibited APC, and that and that in patients with LA both thrombin generation and APC activity were inhibited (Lecompte et al 2007; Regnault et al 2003).

The binding of β_2GPI to anionic phospholipids is dependent on four highly conserved hydrophobic amino acids sequence at positions 313-316. A missense mutation at codon 316 (TGG > TCG) replaces Trp316 with Ser316, resulting in a β_2GPI molecule which is unable to bind phosphatidyl serine (Horbach et al. 1998b; Nash et al. 2003; Rahgozar et al. 2007). I used plasma and immunoglobulin from a patient with anti-β_2GPI antibodies

who was also homozygous for the Trp316Ser mutation (Nash et al 2003) to study the effect of anti-β_2GPI: β_2GPI interactions on thrombin generation and APC function.

My initial work, discussed in the previous chapters, suggested that acquired APC resistance in patients with aPA may be more common than previously thought, and that anti-β_2 glycoprotein-I antibodies were not prerequisite for this phenomenon. The data obtained using the subsampling method showed that the behaviour of aPA was extremely heterogeneous with respect to their action on the procoagulant and natural anticoagulant pathways. I now sought to use automated thrombin generation to study these mechanisms in more detail.

There were several questions that needed to be answered:

- How prevalent were the abnormal thrombin generation patterns that I found using the subsampling method?
- Is abnormal thrombin generation in APS patients antibody mediated, or are there other mechanisms?
- Do monoclonal antibodies react in the same way as affinity purified immunoglobulin from APS patients?
- Do anti β_2GPI antibodies cause resistance to APC, and if so, how?
- Can aPA from a patient homozygous for the Trp316Ser β_2GPI mutation cause APC resistance?
- Can aPA cause resistance to APC independently of β_2GPI?

6.2. Methods

6.2.1. Patients and samples

Venous blood was collected as described in the methods chapter. Plasma from 118 unselected patients, with a range of clinical conditions, sent for antiphospholipid

screening at our laboratory were studied (Table 19). None of the patients had an acute thrombotic event, were receiving anticoagulants or were known to be pregnant at the time of testing. Seven patients with the factor V Leiden mutation were analysed separately, as this mutation has a profound effect on APC sensitivity. Two of these patients had aCL but none had LA or anti-β_2 GPI. Of the remaining 111 patients, 62 were persistently aPA positive (on at least two occasions >12 weeks apart), 40 were negative, and 9 had only transient positivity (i.e., tested on more than one occasion, but not found to be persistently positive). In four asymptomatic patients aPA were found coincidentally to routine coagulation testing. Twenty-five patients fulfilled the modified Sapporo criteria for definite APS (Miyakis et al 2006). For the purposes of this classification, moderate titre anticardiolipin or anti-β_2 GPI antibodies were defined as >15 GPLU or MPLU.

Table 19: Clinical conditions of the patients tested (*some patients had more than one of the listed conditions)

Clinical condition	aPA positive	aPA negative	Transient aPA
Thrombosis			
Arterial	18	3	2
Venous	11	13	1
Pregnancy morbidity			
Recurrent miscarriage (<20 wks)	8	16	2
Intrauterine fetal death (>20 wks)	6	4	1
Preeclampsia	1	2	0
HELLP	3	0	0
Other conditions			
Immune thrombocytopenia	6	0	1
Asymptomatic aPA	4	0	1
Factor V Leiden	3	3	0
Leprosy	1	0	0
Migraine	3	0	0
Leprosy	1	0	0
Systemic lupus erythematosus	2	0	0
Chronic recurrent multifocal osteomyelitis	0	0	1
Immunocompromised	1	0	0
Lymphoproliferative disorder	1	0	0
Family history of thrombosis	1	0	1

6.2.3 In Vitro immunoglobulin spiking experiments

In order to show that the observed impaired response to APC was due to immunoglobulin in aPA patients and, to study aPA antibodies in patients receiving oral anticoagulants, it was first necessary to purify IgG and IgM. To date, very little work has been published on the effects of purified IgM from aPA. Freeze-dried, affinity purified IgG and IgM extracts were dissolved in defibrinated normal pooled plasma at approximately 6mg/ml and 1mg/mL respectively. Doubling dilutions of these preparations were prepared to give 100%, 50%, 25% and 12.5% solutions, which were tested using the ETP and ETP^{+APC} methods. IgG and IgM preparations from normal subjects and aPA positive patients were tested. The clinical and laboratory characteristics are shown in tables 20 and 21.

Table 20: Characteristics of individuals used for preparation of IgG fractions

IgG fraction	Lupus anticoagulant	IgG anticardiolipin	IgG anti-β_2 Glycoprotein-I	Clinical history
N1	-	-	-	none
N2	-	-	-	none
N3	-	-	-	none
aPA 1	+	+	-	Venous thromboembolism
aPA 2	-	++	-	TIA Retinal vein thrombosis
aPA 3	++	+	+++	Venous thromboembolism
aPA 4	++	+++	+++	Stroke preeclampsia
aPA 5	+++	-	-	Stroke SLE TIA
aPA 6	++	++	++	Venous thromboembolism

Table 21: Characteristics of individuals used for preparation of IgM fractions

IgG fraction	Lupus anticoagulant	IgM anticardiolipin	IgM anti-β_2 Glycoprotein-I	Clinical history
N4	-	-	-	none
N5	-	-	-	none
N6	-	-	-	none
aPA7	++	++	++	Venous thromboembolism
aPA8	+++	-	++	Venous thromboembolism
aPA9	+++	+++	+++	Leprosy
aPA10	++	+	+	Stroke preeclampsia

Monoclonal anti-β_2 GPI antibodies (Clone 27671Y; a kind gift from Barry Woodhams, Diagnostica Stago, Asnières, France) were used at 60ug/ml in pooled normal plasma. This clone (27G7) was originally produced and characterised by Arnout *et al* (1998b). This monoclonal antibody demonstrated LA activity in normal plasma (Arnout et al. 1998a) and, although it has been suggested that it may recognise domain I or II of β_2 GPI, the epitope specificity has not been mapped.

6.2.4. β_2 glycoprotein-I experiments

Plasma was obtained from a woman with the Trp316Ser β_2 GPI mutation (Horbach et al 1998b). She had an obstetric history of a stillbirth at 28 weeks, associated with hypertension and severe intrauterine growth restriction and also a miscarriage at 9 weeks. She was persistently positive for IgG anticardiolipin antibodies and IgG antiβ_2-GPI antibodies. The presence of LA was variable, but testing was positive on several occasions. Although the patient has a poor obstetric history, it is uncertain whether these antibodies are pathogenic. Studies examining the binding of the Trp316Ser β_2GPI to

purified cardiolipin showed markedly reduced binding in comparison with β_2 GPI from pooled normal plasma (Nash et al 2003). IgG was purified from this patient as previously described. IgG fractions from a male APS patient with LA, IgG anticardiolipin, IgG anti-β_2 GPI and IgG anti-prothrombin, and pooled normal plasma were also prepared.

ETP and ETP^{+APC} measurements were performed on plasma from the woman with the Trp316Ser β_2 GPI mutation

- ETP and ETP^{+APC} measurements were performed on the following plasma/IgG fraction combinations:
- IgG from the male APS patient was added to pooled normal plasma.
- IgG from the woman with the Trp316Ser β_2GPI mutation was added to pooled normal plasma.
- Normal IgG was added to pooled normal plasma.
- IgG from the male APS patient was added to the plasma of the woman with the Trp316Ser β_2 GPI mutation
- IgG from the woman with the Trp316Ser β_2 GPI mutation

Purified human β_2GPI protein (SCIPAC Ltd, Sittingbourne, UK) was added to the following plasmas at several concentrations (25, 50, 100 and 200 mg/L):

Pooled normal plasma

The plasma of a healthy individual with low plasma β_2 GPI

Plasma from the woman with the Trp316Ser β_2GPI mutation

6.2.5 Antiphospholipid antibody preincubation experiments

In order to investigate the possible specificity of aPA, immunoglobulin fractions were preincubated with plasma, APC and tissue factor prior to measurement of ETP^{+APC}. This was performed using the subsampling endpoint method. I was unable to preincubate antibodies with APC in plasma as APC is rapidly inactivated (Neese, Pratt, & Church 1994). Consequently, it was not possible to examine the effect of anti-β_2GPI and β_2GPI complexes on APC in plasma, prior to the initiation of coagulation.

6.3 Results

6.3.1 Thrombin generation in patients with and without aPA

When all 118 patients were studied, no significant difference in ETP between aPA positive and negative patients was observed. ETP values for aPA positive patients ($p = 0.008$), aPA negative patients and ($p = 0.03$), patients with factor V Leiden ($p = 0.02$) were all significantly higher than the normal healthy subjects (Table 22 and Figure 52). ETP^{+APC} values in aPA positive patients were significantly higher than those in aPA negative patients (Table 22 and Figure 53; $P = 0.02$). All patient groups had significantly higher ETP^{+APC} values than the normal healthy subjects (all $P \leq 0.0001$). It was notable, that the transiently aPA positive patients gave very similar ETP and ETP^{+APC} values to the aPA negative patients, although the numbers were too small to draw any significant conclusions (Tables 22 and 23). If the nAPCsr was used to express the thrombin generation data, no significant differences between aPA negative and positive patients was observed and the transiently positive patients had values similar to the aPA positive patients (Table 23)

In the aPA negative patients high ETP, ETP^{+APC} and nAPCsr values were found in 17/40 (43%), 22/40 (55%) and 18/40 45% respectively, while in the aPA positive patients these figures were 28/62 (45%) 42/62 (68%) and 38/62 (61%). 5/9 (56%), 6/9 (67%) and 6/9 (67%) of the transiently positive patients had abnormal ETP, ETP^{+APC} nAPCsr values. There were no significant differences in the association of aPA status with high ETP, ETP^{+APC} or nAPCsr values, as judged by Fisher's exact test.

Table 22: ETP results in patients with and without aPA
Mann Whitney U test compared with normal subjects ** P < 0.01; § P < 0.05

	Normal healthy subjects (n = 28)	Persistently aPA positive patients (n = 62)	aPA negative patients (n = 40)	Transiently positive aPA patients (n = 9)	Factor V Leiden (n = 8)
Median	0.93	1.05**	1.01§	0.95	1.07§
IQR	0.84 - 1.02	0.85 – 1.22	0.88 – 1.17	0.88 – 0.97	1.00 – 1.11
Minimum	0.73	0.73	0.75	0.75	0.78
Maximum	1.07	1.66	1.10	1.10	1.31

Table 23: ETP^{+APC} results in patients with and without aPA
Mann Whitney U test compared with normal subjects ** P < 0.0001; § P = 0.0001

	Normal healthy subjects (n = 28)	Persistently aPA positive patients (n = 62)	aPA negative patients (n = 40)	Transiently aPA positive patients (n = 9)	Factor V Leiden (n = 8)
Median	0.83	1.46**	1.23§	1.25§	1.84**
IQR	0.73 – 1.00	1.04 – 1.79	1.08 – 1.56	1.08 – 1.56	1.77- 2.32
Minimum	0.41	0.61	0.64	0.89	1.57
Maximum	1.09	2.84	3.38	1.79	3.18

Table 24: nAPCsr results in patients with and without aPA
Mann Whitney U test compared with normal subjects ** P < 0.0001; § P = 0.0001

	Normal healthy subjects (n = 28)	Persistently aPA positive patients (n = 62)	aPA negative patients (n = 40)	Transiently aPA positive patients (n = 9)	Factor V Leiden (n = 8)
Median	0.93	1.42**	1.21§	1.48§	1.77**
IQR	0.84 – 0.93	1.04 – 1.62	0.93 – 1.51	1.22 – 1.61	1.61 – 2.43
Minimum	0.73	1.04	0.65	0.93	1.44
Maximum	1.07	2.90	2.38	1.88	3.14

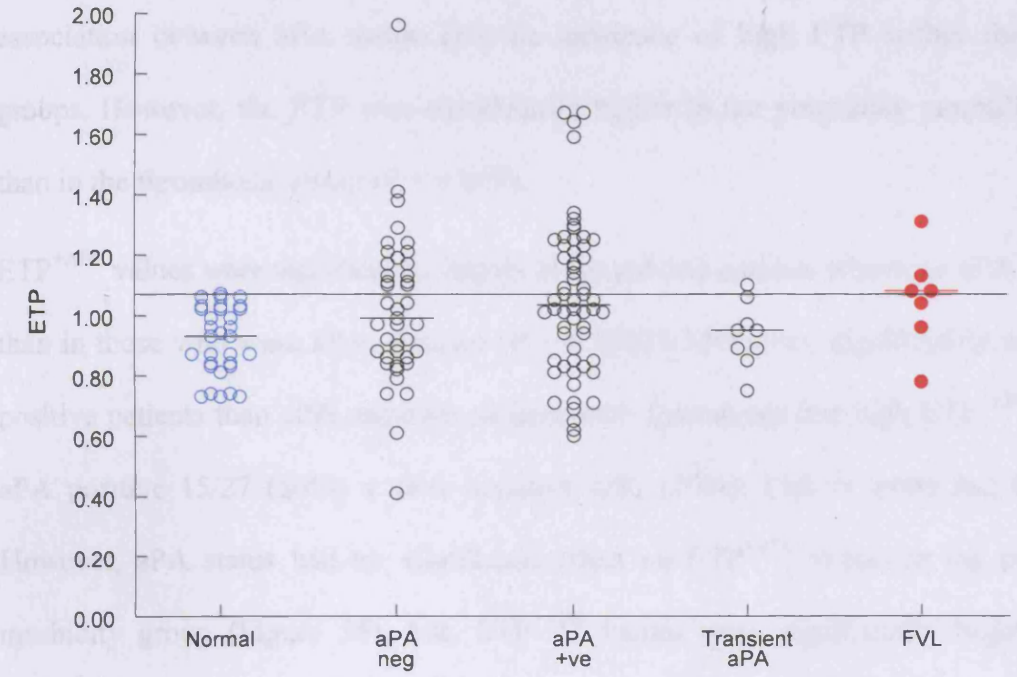

Figure 52: ETP values in normal subjects, aPA negative patients (aPA neg), persistently aPA (aPA +ve), patients with transiently positive aPA, and patients with factor V Leiden (FVL). The broken line indicates the 97.5th percentile of normal.

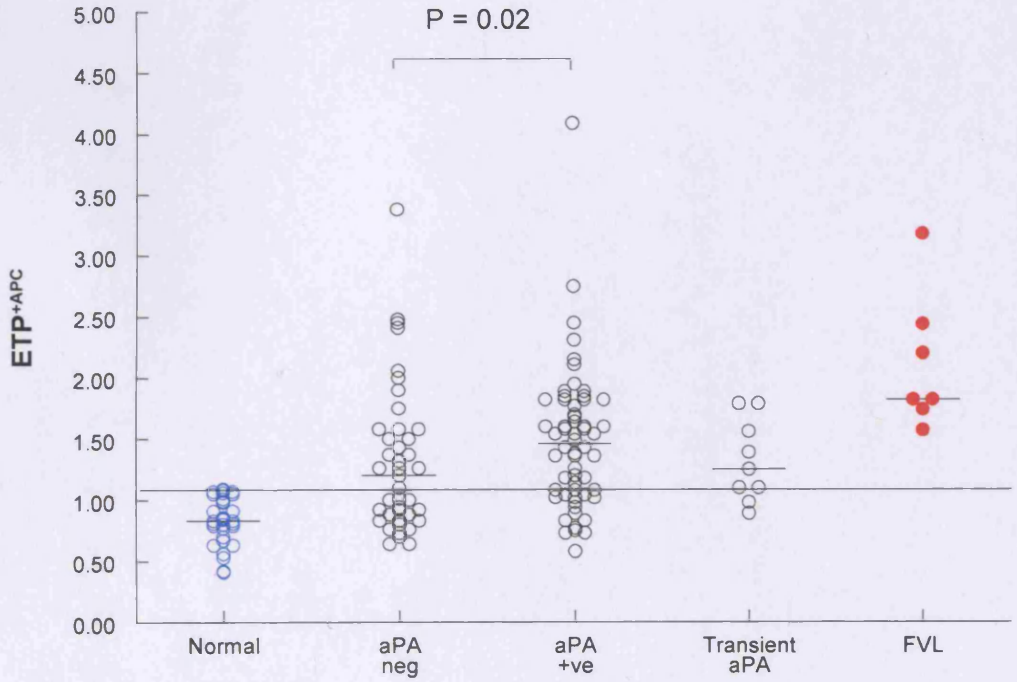

Figure 53: ETP^{+APC} values in normal subjects, aPA negative patients (aPA neg), persistently aPA, patients with transiently positive aPA, and patients with factor V Leiden (FVL) The broken line indicates the 97.5th percentile of normal.

When ETP and ETP^{+APC} were studied by clinical group, aPA status had no significant effect on the median ETP value (Figures 54 and 55). There was no significant association between aPA status and the incidence of high ETP within the clinical groups. However, the ETP was significantly higher in the pregnancy morbidity group than in the thrombosis group (P = 0.007).

ETP^{+APC} values were significantly higher in thrombosis patients who were aPA positive, than in those who were aPA negative (P = 0.0007). Moreover, significantly more aPA positive patients than aPA negative patients with thrombosis had high ETP^{+APC} values: aPA positive 15/27 (56%) v aPA negative 4/21 (19%); Fishers exact test P = 0.01 However, aPA status had no significant effect on ETP^{+APC} values in the pregnancy morbidity group (Figure 55), but, ETP^{+APC} values were significantly higher in the pregnancy morbidity patients than in thrombosis patients (P = 0.001).

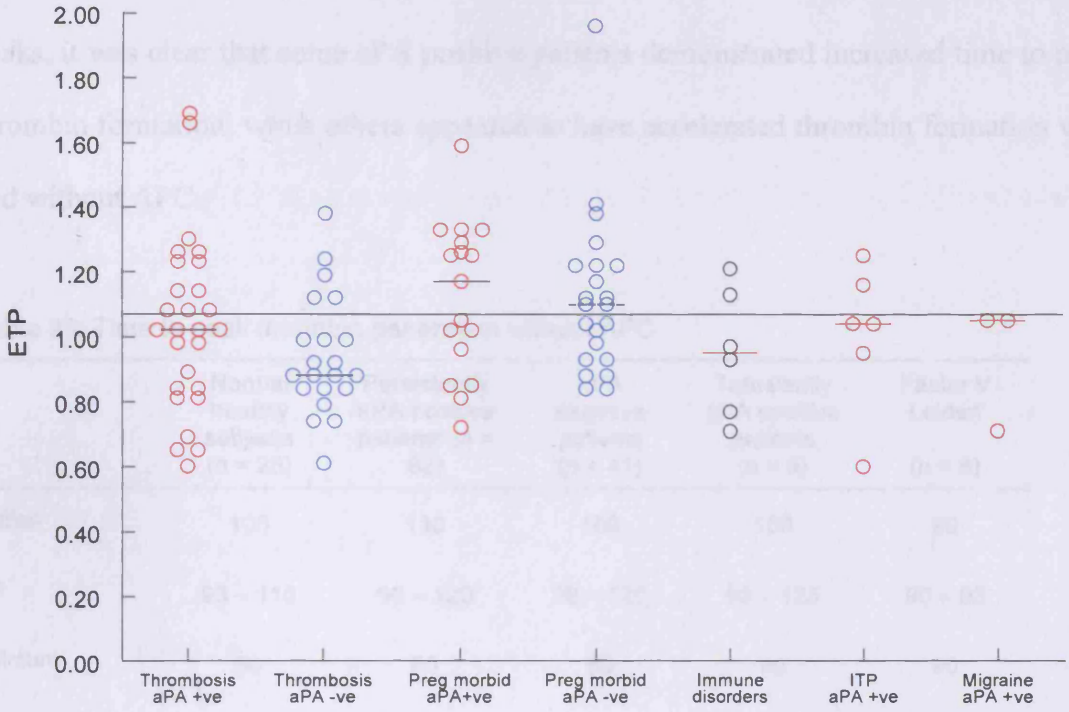

Figure 54: ETP by clinical group

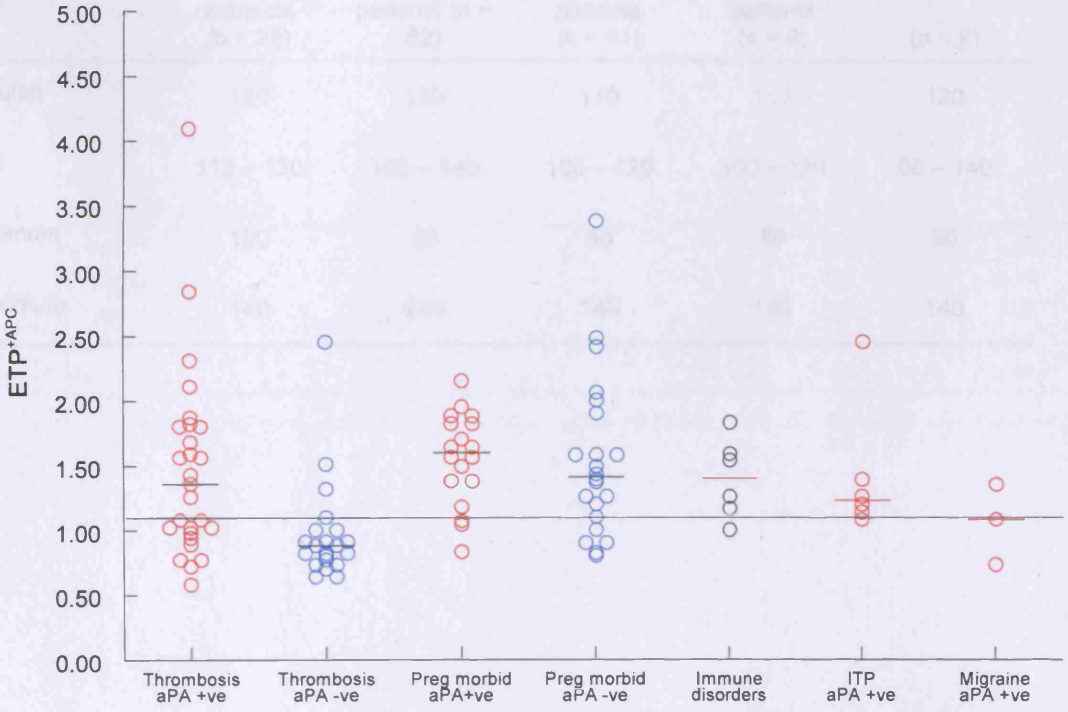

Figure 55: ETP^{+APC} by clinical group.

No significant differences in time to peak thrombin generation, with or without APC were observed between aPA positive and aPA negative patients (Tables 24 and 25). Although it was not always possible to obtain clearly defined thrombin generation peaks, it was clear that some aPA positive patients demonstrated increased time to peak thrombin formation, while others appeared to have accelerated thrombin formation with and without APC.

Table 25: Time to peak thrombin generation without APC

	Normal healthy subjects (n = 28)	Persistently aPA positive patients (n = 62)	aPA negative patients (n = 41)	Transiently aPA positive patients (n = 9)	Factor V Leiden (n = 8)
Median	105	110	100	100	90
IQR	93 – 110	90 – 120	98 – 120	93 – 125	90 – 93
Minimum	90	80	80	90	90
Maximum	120	160	140	140	100

Table 26: Time to peak thrombin generation with APC

	Normal healthy subjects (n = 28)	Persistently aPA positive patients (n = 62)	aPA negative patients (n = 41)	Transiently aPA positive patients (n = 9)	Factor V Leiden (n = 8)
Median	120	130	110	110	120
IQR	113 – 130	100 – 140	100 – 120	100 – 120	98 – 140
Minimum	100	80	80	80	90
Maximum	140	210	140	140	140

Of the 25 patients who met the revised Sapporo criteria (Miyakis et al 2006), twenty (80%) had a raised nAPCsr, thirteen (52%) had raised ETP and nineteen (76%) had raised ETP^{+APC} (Table 27). There was no obvious association between ETP or ETP^{+APC} and clinical presentation, LA, aCL or anti-β_2GPI positivity.

Table 27: Characteristics of patients meeting the Sapporo criteria for definite antiphospholipid syndrome.

Patient	Clinical presentation	LA	IgG	IgM	β_2 IgG	β_2 IgM	nAPCsr	ETP	ETP^{+APC}
1	TIA + arterial stroke	+	-	++	-	+++	0.88	1.01	0.89
2	Arterial stroke	-	+++	-	-	-	1.00	0.94	0.94
3	DVT	++	-	+++	-	++	1.16	0.84	0.98
4	DVT	++	-	-	-	++	0.93	1.08	1.01
5	Arterial stroke	+	++	-	-	-	0.80	1.30	1.04
6	RM	-	++	+	-	+	1.26	0.85	1.07
7	ITP TIA + arterial stroke	+++	-	-	-	-	2.10	0.60	1.26
8	Recurrent DVT + PE	+	++	-	+	-	1.64	0.83	1.36
9	RM	-	++	-	-	-	1.61	0.85	1.37
10	Ret vein thrombosis + TIA	-	++	-	-	-	1.26	1.14	1.43
11	DVT	++	++	-	-	-	1.72	0.89	1.54
12	DVT	-	++	-	-	-	2.24	0.69	1.56
13	Preeclampsia	-	++	-	-	-	1.29	1.23	1.58
14	RM	+	-	-	-	-	1.24	1.32	1.64
15	TIA	++	-	-	-	-	1.37	1.23	1.68
16	CI 2° to arterial disease	++	-	-	-	+	1.49	1.19	1.77
17	TIA + arterial stroke	+++	-	++	-	-	2.79	0.65	1.81
18	TIA + arterial stroke	++	+	-	-	++	1.44	1.26	1.82
19	RM + preeclampsia	-	-	-	+++	-	1.58	1.17	1.85
20	IUFD + preeclampsia	+	+	-	-	+	1.51	1.29	1.95
21	CI 2° to arterial disease	+++	+	-	-	-	1.94	1.09	2.11
22	RM	+	-	-	-	-	1.62	1.33	2.15
23	DVT	+++	+	+	-	++	2.90	0.80	2.31
24	DVT	++	-	-	-	-	1.72	1.66	2.84
25	CI 2° to arterial disease	+	+++	-	+	+	2.45	1.67	4.09
	Median						1.51	1.09	1.58
	25th percentile						1.26	0.85	1.26
	75th percentile						1.72	1.26	1.85
	Minimum						1.24	0.65	1.26
	Maximum						2.90	1.67	4.09

CI = cerebral ischaemia; DVT = deep vein thrombosis; TIA = transient ischaemic attacks; RM = recurrent miscarriage; PE = pulmonary embolism

6.3.2 The effect of IgG and IgM from patients with aPA on thrombin generation

IgG fractions prepared from three normal healthy subjects and five patients with APS, were added to pooled normal plasma to examine the effect on ETP and ETP^{+APC}. ETP was reduced by 4/5 IgG fractions, while ETP^{+APC} was increased by 3/5 fractions (Table 28; Figures 56 and 57). It was notable that fraction aPA5 (a patient with no detectable anti-β_2GPI) inhibited thrombin generation in the absence of APC but also inhibited APC activity. The time to peak thrombin generation with and without APC was prolonged by both aPA5 (Figure 58) and aPA6.

Table 28: The effect of IgG fractions on ETP and ETP^{+APC}

IgG fraction	IgG conc (mg/ml)	nAPCRsr	ETP	ETP $+^{APC}$
N1	4.3	1.05	0.99	1.04
N2	3.7	1.07	0.89	0.95
N3	4.1	1.00	0.88	0.88
aPA 1	5.3	1.02	0.96	0.98
aPA 2	6.0	1.03	0.86	0.89
aPA 3	4.5	1.08	0.92	0.99
aPA 4	4.3	1.20	0.89	1.07
aPA 5	6.7	2.30	0.76	1.75
aPA 6	6.9	2.29	0.78	1.79

IgM fractions prepared from three normal healthy subjects and four patients with APS, were added to pooled normal plasma to examine the effect on ETP and ETP^{+APC}. ETP was reduced and ETP^{+APC} was increased by all four IgM fractions from patients with APS. (Table 29; Figures 56 and 57).

Table 29: The effect of IgM fractions on ETP and ETP^{+APC}

IgM fraction	IgM conc (mg/ml)	nAPCRsr	ETP	ETP $+^{APC}$
N4	1.1	1.09	0.99	1.08
N5	1.5	1.10	0.96	1.06
N6	1.2	1.24	0.96	1.04
aPA7	1.1	1.56	0.82	1.28
aPA8	0.6	1.69	0.78	1.32
aPA9	1.6	2.51	0.53	1.33
aPA10	0.7	2.82	0.62	1.75

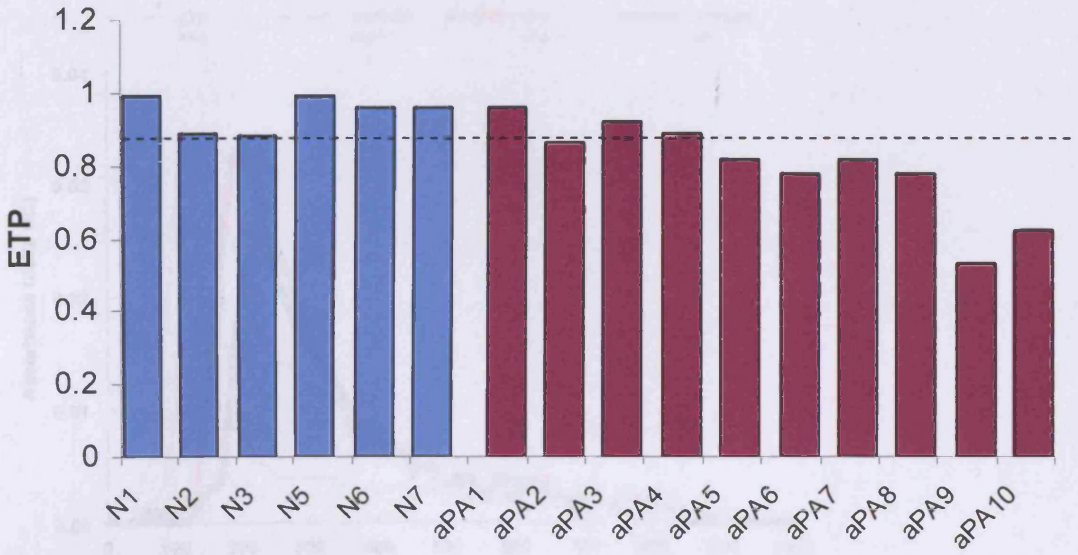

Figure 56: The effect of the addition of purified immunoglobulin to normal plasma on ETP. The broken line indicates the normal lower limit.

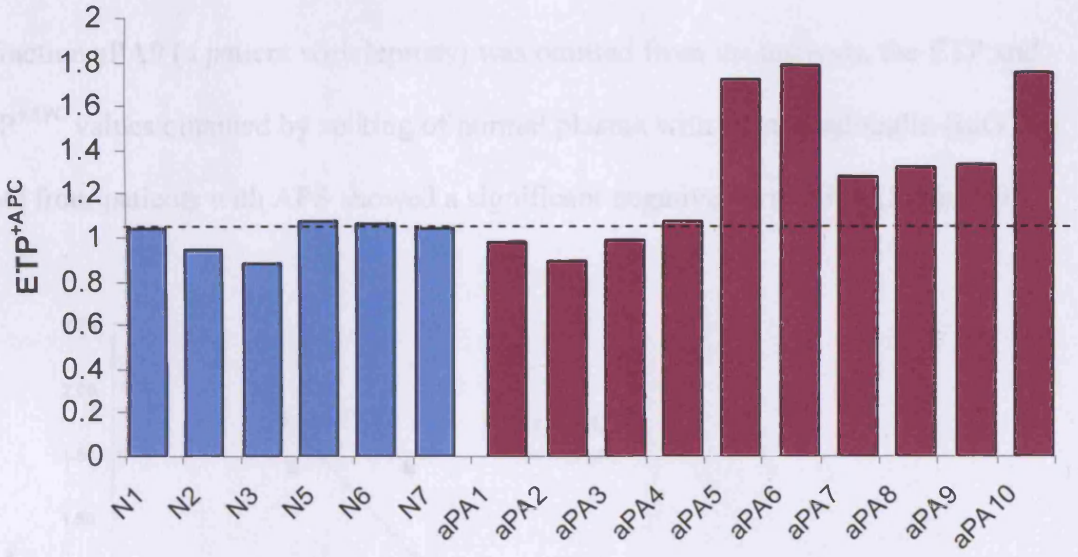

Figure 57: The effect of the addition of purified immunoglobulin to normal plasma on ETP^{+APC}. The broken line indicates the normal upper limit.

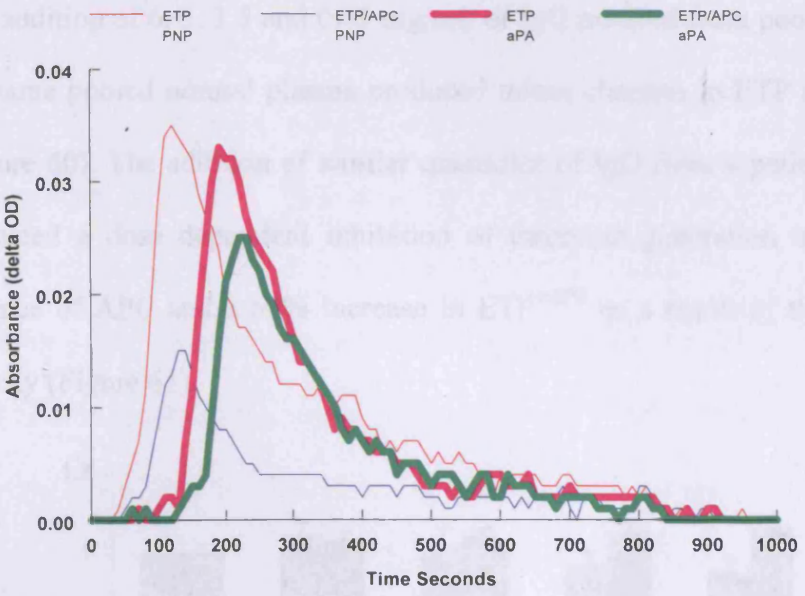

Figure 58: The effect of 6.7 mg/ml purified IgG from a patient (aPA5) with antiphospholipid antibodies on normal plasma

If fraction aPA9 (a patient with leprosy) was omitted from the analysis, the ETP and ETP^{+APC} values obtained by spiking of normal plasma with immunoglobulin (IgG and IgM) from patients with APS showed a significant negative correlation (Figure 59).

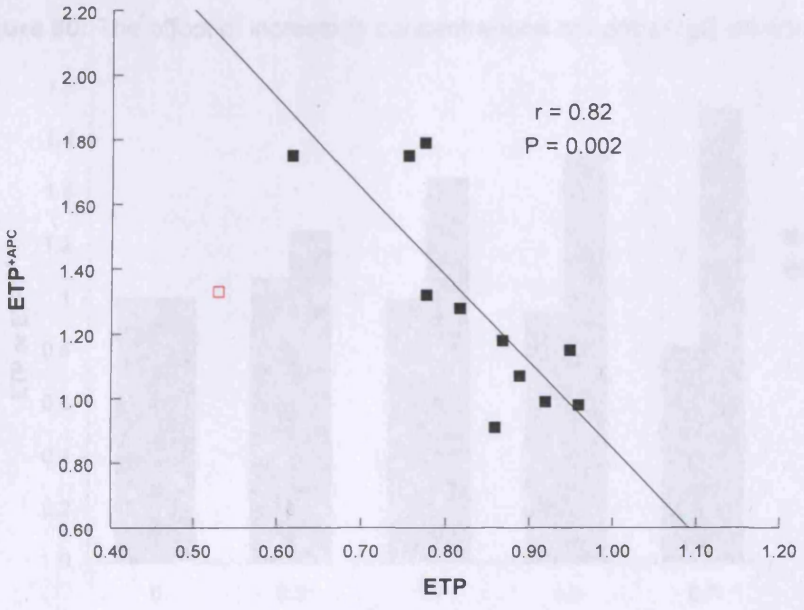

Figure 59: The effect of the addition of purified immunoglobulin to normal plasma on the relationship between ETP and ETP^{+APC}.
The point shown in red was from a patient with immune IgM secondary to leprosy and was excluded from the analysis.

The addition of 6, 3, 1.5 and 0.75 mg/mL of IgG purified from pooled normal plasma to the same pooled normal plasma produced minor changes in ETP and ETP^{+APC} of <6% (Figure 60). The addition of similar quantities of IgG from a patient with APS (aPA5) produced a dose dependent inhibition of thrombin generation of up to 23% in the absence of APC and a 59% increase in ETP^{+APC} as a result of the inhibition of APC activity (Figure 61).

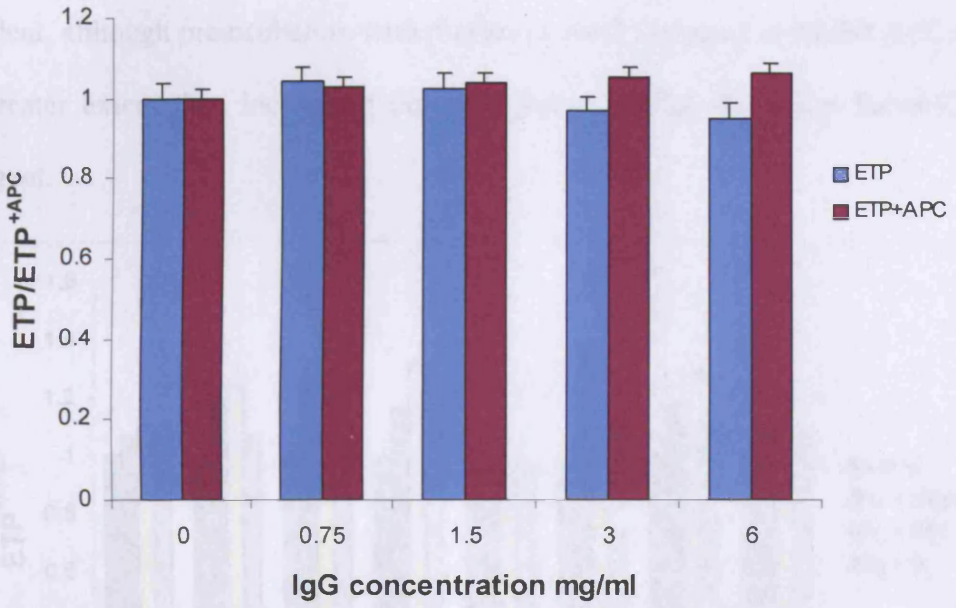

Figure 60: The effect of increasing concentrations of normal IgG on normal plasma (n = 3)

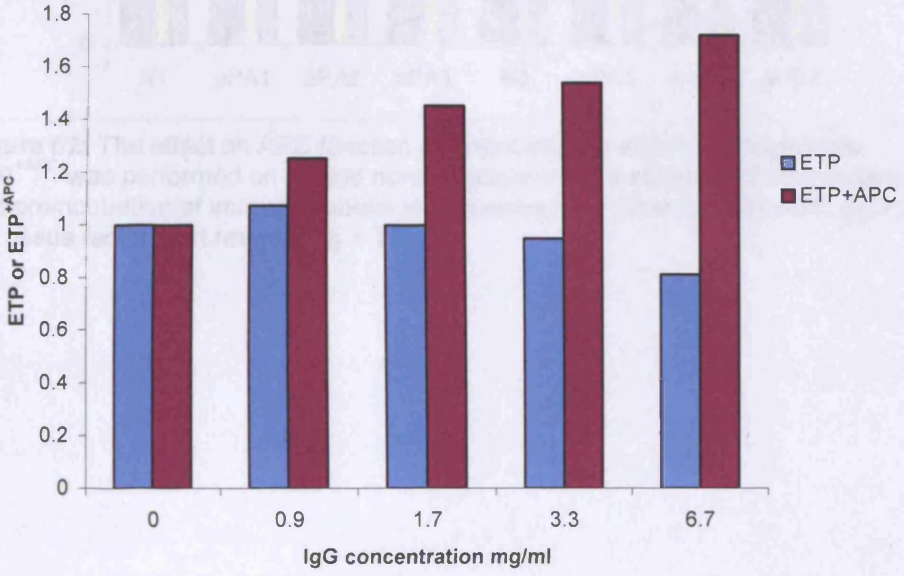

Figure 61: The effect of purified IgG from a patient with APS (aPA5) on normal plasma

6.3.3 The effect on APC function of preincubation with immunoglobulin

In order to investigate the possible specificity of aPA, immunoglobulin fractions from patients with APS were preincubated with plasma, APC and tissue factor prior to measurement of ETP^{+APC}. The addition of purified immunoglobulin increased ETP^{+APC} as previously demonstrated. In aPA fractions 1, 3, 7 and 9, this was more pronounced if the aPA were first incubated with APC, while in aPA fractions 2 and 6, preincubation with plasma produced the largest increases in ETP^{+APC} (Figure 62). No clear pattern was evident, although preincubation with plasma or APC appeared to inhibit APC activity to a greater extent than incubating the aPA fractions with the tissue factor/$CaCl_2$ start reagent.

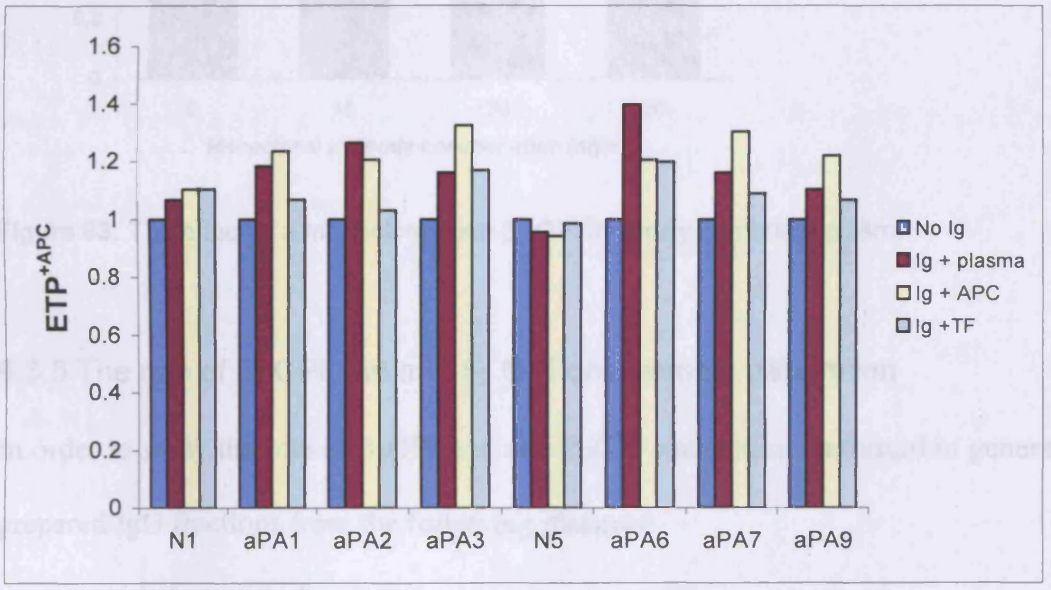

Figure 62: The effect on APC function of preincubation with immunoglobulin. ETP^{+APC} was performed on pooled normal plasma in the absence of immunoglobulin (No Ig), and preincubation of immunoglobulin with plasma (Ig + plasma), with APC (Ig + APC) and with the tissue factor start reagent (Ig + TF).

6.3.4. The effect of anti β_2 GPI monoclonal antibodies on thrombin generation

The addition of increasing amounts of a monoclonal anti-β_2GPI antibody (27G7) with LA activity (Arnout et al 1998a) to pooled normal plasma caused minor decreases in ETP of up to 9% and a dose-dependent increase in ETP^{+APC} of up to 16% (Figure 63).

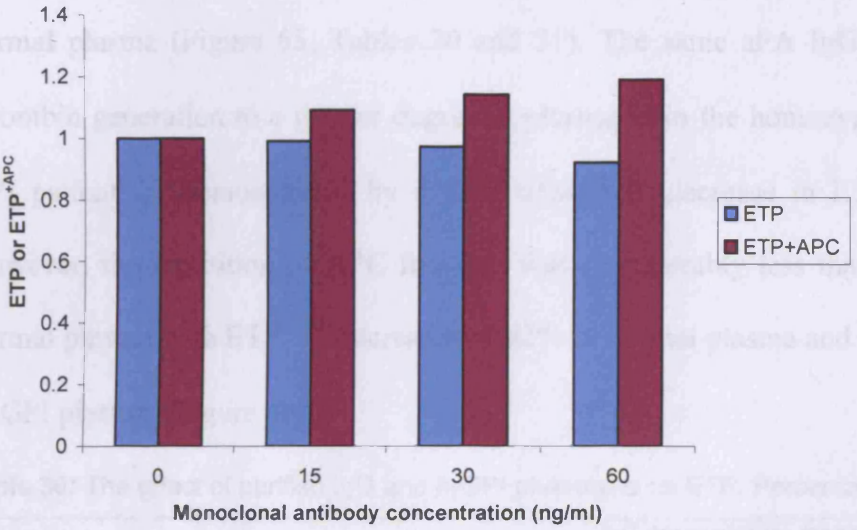

Figure 63: The effect of a monoclonal anti-β_2 GPI antibody on normal plasma

6.3.5 The role of β_2 GPI and anti-β_2 GPI on thrombin generation

In order to study the role of β_2GPI and anti-β_2GPI interaction on thrombin generation, I prepared IgG fractions from the following plasmas:

- pooled normal plasma
- a patient homozygous for the Trp316Ser β_2GPI mutation with anti-β_2GPI
- a patient with potent aPA and wild-type β_2GPI (aPA6)

These were added to normal plasma (wild type β_2GPI) and to plasma from the patient homozygous for the Trp316Ser β_2 GPI mutation.

ETP and ETP^{+APC} values for plasma from the Trp316Ser patient and a 50:50 mix with normal plasma were very similar (ETP 1.25 and 1.20; ETP^{+APC} 0.83 and 0.87 respectively). Normal IgG produced minor changes in ETP and ETP^{+APC} as previously

shown (Figure 60). The IgG purified from the patient with anti-β_2 GPI and homozygous for the Trp316Ser mutation also caused minor reductions ETP and ETP^{+APC} (<10%) (Figure 64, Tables 30 and 31).

IgG from a patient with potent LA and anti-β_2 GPI (aPA6) caused a dose dependent decrease in ETP of up to 14% and dose dependent increases in ETP^{+APC} of up to 98% in normal plasma (Figure 65, Tables 30 and 31). The same aPA IgG fraction inhibited thrombin generation to a greater degree in plasma from the homozygous Trp316Ser β_2 GPI patient, as demonstrated by a dose dependent decrease in ETP of up to 45%. However, the inhibition of APC function was considerably less than that observed in normal plasma with ETP^{+APC} increases of 82% in normal plasma and 52% in Trp316Ser β_2 GPI plasma (Figure 4b).

Table 30: The effect of purified IgG and β_2GPI phenotype on ETP. Percentage change

Plasma β_2GPI	IgG source	IgG concentration mg/ml		
		1.5	3.0	6.0
Wild type	Normal plasma	+2%	-3%	-5%
Wild type	aPA positive Trp316Ser patient	-1%	-6%	-10%
Wild type	aPA6	-4%	-3%	-14%
TrpSer316	aPA6	-11%	-30%	-45%

Table 31: The effect of purified IgG and β_2GPI phenotype on ETP^{+APC}. Percentage change

Plasma β_2GPI	IgG source	IgG concentration mg/ml		
		1.5	3.0	6.0
Wild type	Normal	+4%	+5%	+6%
Wild type	aPA positive Trp316Ser patient	+1%	-3%	-5%
Wild type	aPA6	+55%	+82%	+98%
TrpSer316	aPA6	+35%	+52%	+55%

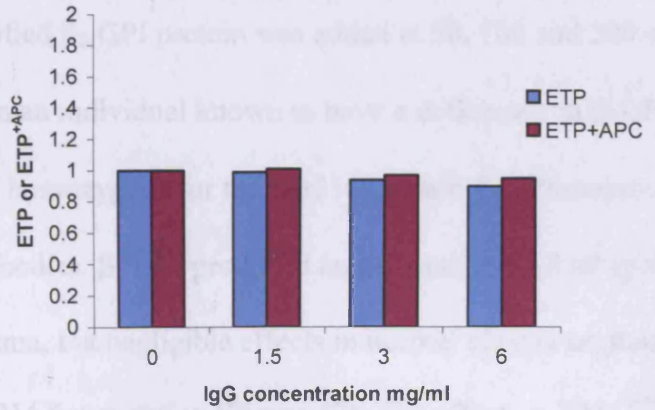

Figure 64: The effect of purified IgG from a patient with aPA and the Trp-Ser β_2GPI mutation on wild type plasma

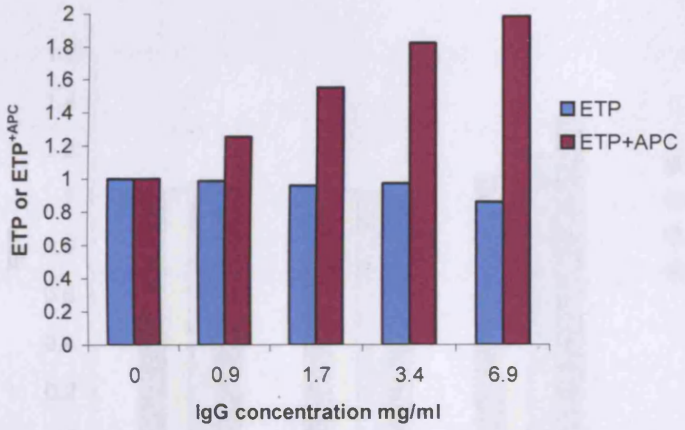

Figure 65: The effect of purified IgG from a patient with aPA (aPA6) on wild type plasma

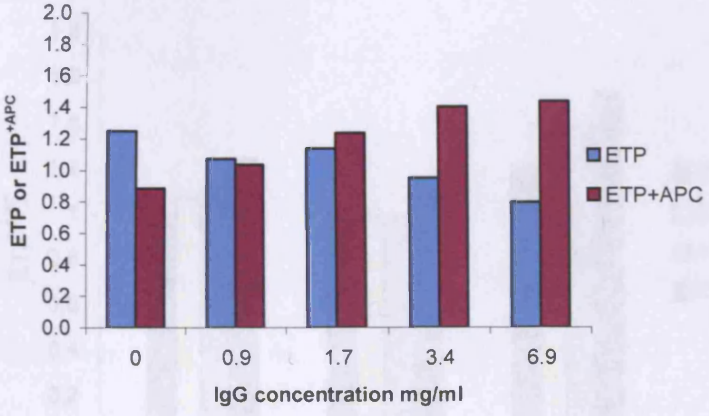

Figure 66: The effect of purified IgG from a patient with aPA (aPA6) on plasma from a patient homozygous for the Trp316Ser β_2 GPI mutation

Purified β_2 GPI protein was added at 50, 100 and 200 mg/mL to normal plasma, plasma from an individual known to have a deficiency in β_2GPI and plasma from a patient who was homozygous for the Ser316Trp anti-β_2GPI mutation who also had anti-β_2GPI antibodies. β_2 GPI produced an increase in ETP of up to 27% in the β_2GPI deficient plasma, but negligible effects in normal plasma or plasma from the patient with the Ser316Trp mutation (Figure 67). The effect on ETP^{+APC} was more pronounced with dose dependent responses of up to 20%, 12% and 21% for normal, Ser316Trp plasma and β_2GPI deficient plasma respectively (Figure 68).

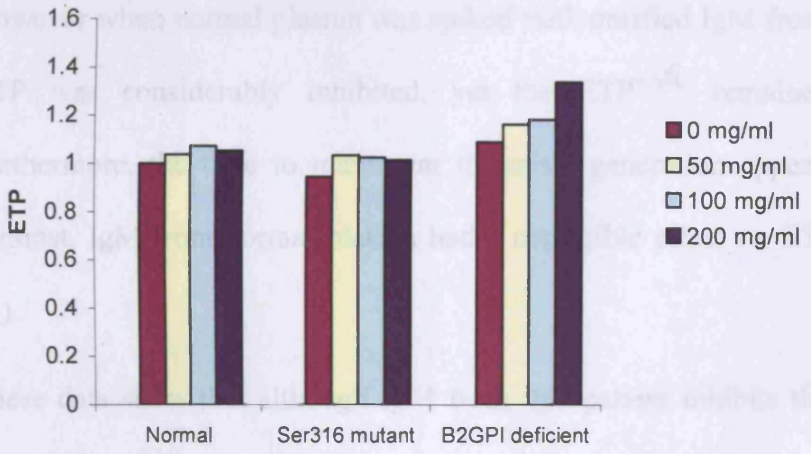

Figure 67: The effect of added β_2GPI concentration on ETP in wild type, homozygous Ser316Trp β_2GPI, and β_2GPI deficient plasmas

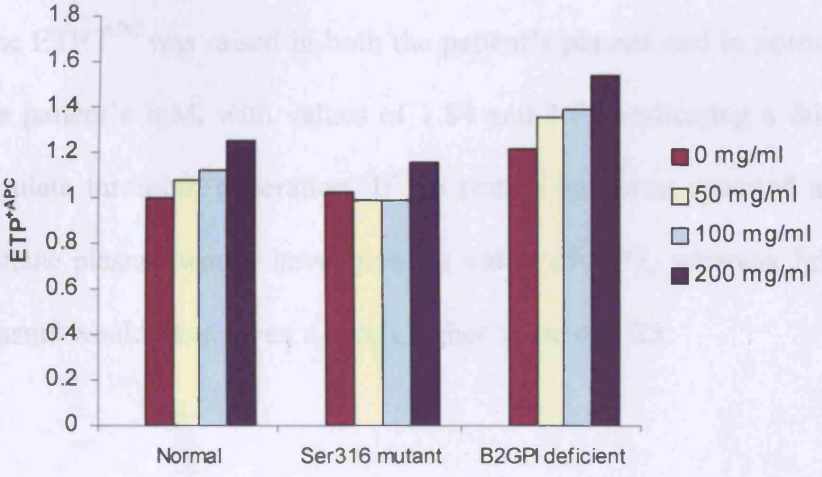

Figure 68: The effect of added β_2GPI concentration on ETP^{+APC} in wild type, homozygous Ser316Trp β_2GPI, and β_2GPI deficient plasmas

6.3.6 A study of a patient with leprosy and aPA

I studied one patient with leprosy in detail. This patient had strong LA activity and an IgM anti-β_2 GPI titre of >1000 MPLU, but no IgG aCL or IgG anti-β_2 GPI. He also had a severe acute phase response with a factor VIII:C level of >400 IU/dL, C reactive protein of 152 mg/mL, an erythrocyte sedimentation rate of 44 mm/hour and acquired APC resistance by the APTT based method (modified APC ratio 1.52, factor V Leiden normal). His free protein S level was 0.22 IU/mL, which may have been due to high levels of C4b binding protein.

Both ETP and ETP^{+APC} were increased relative to that of normal plasma (Figure 69). However when normal plasma was spiked with purified IgM from the same patient, the ETP was considerably inhibited, yet the ETP^{+APC} remained high (Figure 70). Furthermore, the time to maximum thrombin generation appeared to be delayed. In contrast, IgM from normal plasma had a negligible effect on ETP or ETP^{+APC} (Figure 71).

These data show that although IgM from this patient inhibits thrombin generation (in the absence of APC), the underlying acute phase response produces an excess of thrombin generation, thus masking the inhibitory effect of the IgM on ETP.

The ETP^{+APC} was raised in both the patient's plasma and in normal plasma spiked with the patient's IgM, with values of 1.84 and 1.71, indicating a failure of APC to down regulate thrombin generation. If the results had been reported as nAPCsr values, the patient plasma would have given a value of 1.57, whereas IgM spiking of normal plasma would have given a much higher value of 3.25.

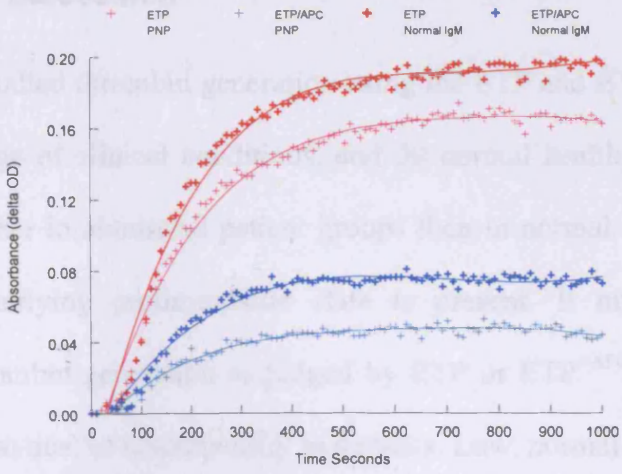

Figure 69: Thrombin generation in a patient with immune anti-β_2 GPI secondary to leprosy infection.

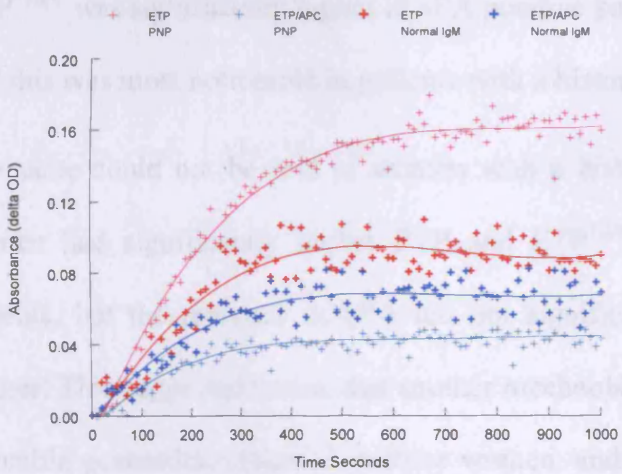

Figure 70: Thrombin generation in normal plasma with added IgM from a patient with immune anti-β_2 GPI secondary to leprosy infection (aPA 9).

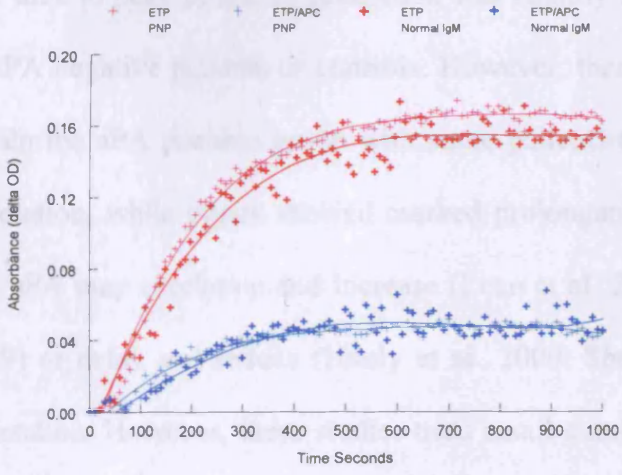

Figure 71: Thrombin generation in normal plasma with added IgM from a normal healthy subject (N6)

6.4 Discussion

I studied thrombin generation using the ETP and ETP^{+APC} assays in 118 patients with a range of clinical conditions, and 30 normal healthy subjects. ETP and ETP^{+APC} were higher in almost all patient groups than in normal healthy subjects, suggesting that an underlying prothrombotic state is present. It must be emphasised that increased thrombin generation as judged by ETP or ETP^{+APC} was not a uniform finding with a great deal of heterogeneity in patients. Low, normal and raised ETP and ETP^{+APC} values were all observed. Furthermore, aPA status had no significant effect on the ETP value, which would imply that aPA does not increase thrombin generation *per se*. However, ETP^{+APC} was significantly higher in aPA positive patients than in aPA negative patients, and this was most noticeable in patients with a history of thrombosis.

The same could not be said of women with a history of pregnancy morbidity. These women had significantly higher ETP and ETP^{+APC} values than any other group of patients, but the presence of aPA did not significantly increase thrombin generation further. This suggested to me, that another mechanism was responsible for the increased thrombin generation observed in these women, and this was investigated further in the next chapter.

The time to peak thrombin generation was slightly longer in aPA positive patients than in aPA negative patients or controls. However, there was a great deal of heterogeneity within the aPA positive group with some patients demonstrating accelerated thrombin generation, while others showed marked prolongation. It has previously been reported that aPA may accelerate and increase (Lean et al 2006; Liestol et al 2007; Rand et al 1999) or delay and reduce (Hanly et al 2000; Sheng et al 2001a) *in vitro* thrombin generation. However, these studies used small numbers of highly selected patients. My data was obtained from a large number of patients with a wide range of clinical conditions, and these show that the thrombin generation phenotype in APS is much

more heterogeneous than previously thought, with a wide spectrum of thrombin generation levels and variable resistance to APC.

When I spiked plasma with IgG and IgM from patients with APS, the trend was towards decreased ETP and increased ETP^{+APC}. As may have been expected, immunoglobulin fractions from moderate to strongly LA positive patients inhibited thrombin generation to the greatest degree. These fractions also appeared to delay the onset of thrombin generation and increased the time taken to peak thrombin formation as previously reported (Regnault et al 2003). That these immunoglobulin fractions should be responsible for the most pronounced APC resistance was less expected. Furthermore, ETP and ETP^{+APC} showed a significant negative correlation. In contrast to previous published reports, anti-β_2GPI was not prerequisite for APC resistance (Galli et al 1998) (Martinuzzo et al 1996; Mercier et al 1998; Viveros et al 2005), as not all fractions containing anti-β_2GPI produced APC resistance, and a fraction from a patient with no detectable anti-β_2GPI strongly inhibited APC. I concluded it was the ability of antibodies with LA activity to prevent complex formation of phospholipid-binding proteins on negatively charged phospholipid rather than anti-β_2GPI specificity *per se* that was most likely responsible for acquired APC resistance in APS. This is consistent with the finding bivalent aPA enhance binding of prothrombin to negatively charged phospholipid and inhibit APC (Field et al. 2001; Field, Chesterman, & Hogg 2000)

In order to test this theory further, I used plasma and IgG from a patient homozygous for the non-binding form of β_2GPI (Trp316Ser) who had anti-β_2GPI and LA activity.

It has been suggested that phospholipid binding of β_2GPI to negatively charged phospholipids causes a conformational change and revealing a neoepitope (Kurosawa, Esmon, & Stearns-Kurosawa 2000; Kuwana et al. 2005; Matsuura et al 1994; Subang et al. 2000). As Trp316Ser β_2GPI is non-binding, it has been proposed that individuals

who are homozygous for the Trp316Ser mutation may be protected against the development of anti-β_2GPI antibodies (Horbach et al 1998b). However, anti-β_2GPI antibodies have been reported in this patient homozygous for the Trp316Ser mutation (Nash et al 2003).

This patient's plasma and 50:50 mix in normal plasma gave very similar values for ETP and ETP^{+APC}, suggesting that the patient's anti-β_2GPI antibodies had a negligible effect on normal β_2GPI. IgG from the patient produced only minor changes in ETP and ETP^{+APC} when added to normal plasma, again suggesting that the patient's IgG was not interacting with the normal β_2GPI. In contrast, when IgG from a patient with potent aPA (anti-β_2GPI and LA activity) was added to the Trp316Ser plasma in the absence of APC, thrombin generation was inhibited to a greater degree than observed in normal plasma. The reason for this is unclear, but it cannot be explained by enhanced binding of a non-binding β_2GPI. As the patient had a high titre of anti-prothrombin antibodies, this would seem to be an obvious candidate, as anti-prothrombin antibodies have been shown to inhibit APC function in the presence of calcium (Field et al 2000; Galli et al 2005). The addition of normal β_2GPI to the Trp316Ser plasma did not significantly affect ETP or ETP^{+APC}.

It would appear that anti-β_2GPI antibodies produced in this patient, homozygous for the Trp316Ser β_2GPI mutation, are incapable of inhibiting APC even if normal β_2GPI is added, but that exogenous aPA from a patient with normal β_2GPI may cause APC resistance in an apparently β_2GPI-independent manner.

The same aPA positive IgG fraction caused an increase in ETP^{+APC} in Trp316Ser plasma of approximately 50%; roughly half that observed in normal plasma, thus demonstrating that while acquired APC resistance may be caused by anti-β_2GPI - β_2GPI interactions, other mechanisms, independent of β_2GPI are also present. These data seem

to support the neoepitope theory (Matsuura et al 1994; Pengo, Biasiolo, & Fior 1995; Subang et al 2000), rather increased antigen density being responsible for binding of aPA to β_2GPI on negatively charged surfaces (Sheng et al 1998).

Thrombin generation in the plasma from the leprosy patient was very different to that of pooled normal plasma spiked with IgM from the same patient. This patient's IgM strongly inhibited APC activity when added to normal plasma. However, the patient had a severe acute phase response, resulting in raised ETP, and this masked the inhibitory effect of the aPA on thrombin generation. Clearly, in these situations, the acute phase response may have more effect than aPA on thrombin generation. This, in my opinion, justified my decision to study patients only after the acute clinical phase had resolved, during this work.

In conclusion, I have demonstrated variable thrombin generation and response to APC in patients with APS. I have shown that APC resistance is closely associated with LA and, while this may be due in part to anti-β_2GPI antibodies, β_2GPI independent mechanisms also exist. The prolongation of the time to peak thrombin generation by aPA is analogous to the increased clotting time observed in patients with LA. I have shown that LA activity is strongly associated with a resistance to APC and that, while LA may delay and reduce thrombin generation in the absence of APC, in the presence of APC an excess of thrombin is formed. These data go some way to explaining the lupus anticoagulant paradox, and the strong association between LA and thrombosis (Galli et al 2003b).

Chapter 7. Acquired APC resistance, TFPI and adverse obstetric events

7.1. Introduction

Recurrent pregnancy loss, is a common problem, with two or more losses in up to 5% of women, and three or more losses affecting 1-2% of women (Sarig et al. 2005). The association of a circulating anticoagulant and pregnancy loss was first described by Nilsson et al (1975), who reported the presence of "antithromboplastin" in a woman who had three interuterine deaths characterised by placental infarction. Markers of coagulation activation (D-dimer and fibrinopeptide A) are known to increase preceding spontaneous abortion (Woodhams et al. 1989). There is a strong association with both aPA (Rai et al. 1995) and to a lesser extent congenital thrombophilia (Preston et al. 1996). In women with recurrent miscarriage due to aPA, the fetal loss rate may be as high as 90% (Rai et al 1995) with the majority of these occurring in the first trimester. The picture with heritable thrombophilia is not so clear, with highly variable associations depending upon the defect concerned (Robertson et al 2006)

In the previous chapter, I showed that while ETP or ETP^{+APC} were raised in many women with a history of pregnancy morbidity, aPA status appeared to have no significant effect. I decided to study an extended cohort of women with a history of pregnancy morbidity, to see if this was a genuine finding rather than simple a type 2 statistical error. As I demonstrated in Chapter 5, TFPI is an important determinant of ETP-based APC sensitivity based assays such as ETP^{+APC}, so I also investigated TFPI antigen, activity and the frequency of anti-TFPI antibodies in this group, to ascertain whether these factors could explain the increased thrombin generation and resistance to APC seen in these patients. I also investigated the effect of heparin on plasma TFPI levels and the relationship between TFPI, thrombin generation and resistance to APC.

7.2 Methods

7.2.1 Patients and samples

I studied thrombin generation, plasma TFPI antigen and activity and anti-TFPI antibodies in an extended group of 52 women (including the 44 women from the previous chapter) with a history of recurrent miscarriage (33), intrauterine fetal death (13), preeclampsia (3) or the HELLP syndrome (haemolysis with elevated liver enzymes and low platelets, 3). None had known heritable thrombophilia but twenty had persistent aPA and six had transient aPA positivity. All samples were taken at least six weeks after the end of pregnancy.

Total TFPI antigen was assayed using the IMUBIND® Total Tissue Factor Pathway Inhibitor ELISA kit (American Diagnostica) and the TFPI sensitivity index used the method of Dahm *et al* (2005) as described in the methods chapter.

7.3 Results

7.3.1 Thrombin generation and pregnancy morbidity

Both ETP and ETP^{+APC} were significantly higher than in women with previous pregnancy morbidity than normal women (Table 32; Figure 72). Raised ETP and ETP^{+APC} values were obtained in 27/52 (52%) and 41/52 (79%) of women respectively. Only 8/52 (15%) had normal thrombin generation by both methods. However, as with the smaller series studied in the previous chapter, the antiphospholipid status of the women had no significant effect on ETP or ETP^{+APC}. When the data from with pregnancy loss were analysed by time of loss, again there was no significant effect on ETP or ETP^{+APC} (Table 32; Figure 73).

Table 32: Thrombin generation and TFPI measurements in women with a history of pregnancy morbidity: Median (interquartile range)

Patient group	ETP	ETP^{+APC}	TFPI Ag (ng/ml)	TFPI Index
Female reference range (n =20)	0.76 – 1.07	0.77 – 1.06	75 - 120	0.96 – 1.01
aPA positive aPA (n = 20)	1.14 (0.78 – 1.47)	1.59 (1.03 – 2.06)	72 (46 – 102)	0.96 (0.91 – 1.07)
Transient aPA positive (n = 6)	0.94 (0.86 – 0.98)	1.09 (0.99 – 1.23)	74 (54 –98)	0.96 (0.93 – 0.97)
aPA negative (n = 26)	1.10 (0.94 – 1.22)	1.46 (1.21 – 1.81)	83 (73 – 103)	0.96 (0.92 – 0.97)
Recurrent miscarriage (n = 33)	1.10 (0.83 – 1.66)	1.56 (0.82 – 2.66)	85 (37 – 118)	0.96 (0.89 – 1.06)
IUFD (n = 13)	1.21 (0.65 – 1.33)	1.43 (1.00 – 1.94)	69 (35 – 104)	0.96 (0.94 –1.02)
Preeclampsia (n = 3)	0.93	1.49	74	0.94
HELLP (n = 3)	1.01	1.38	105	0.94

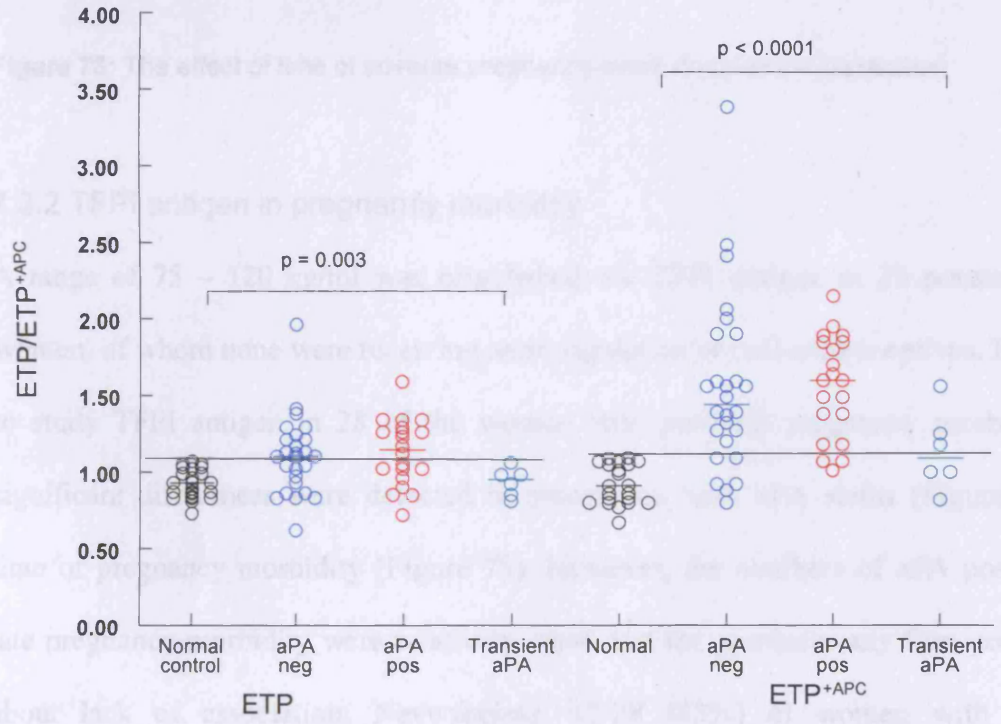

Figure 72: The effect of aPA status on thrombin generation in women with pregnancy morbidity

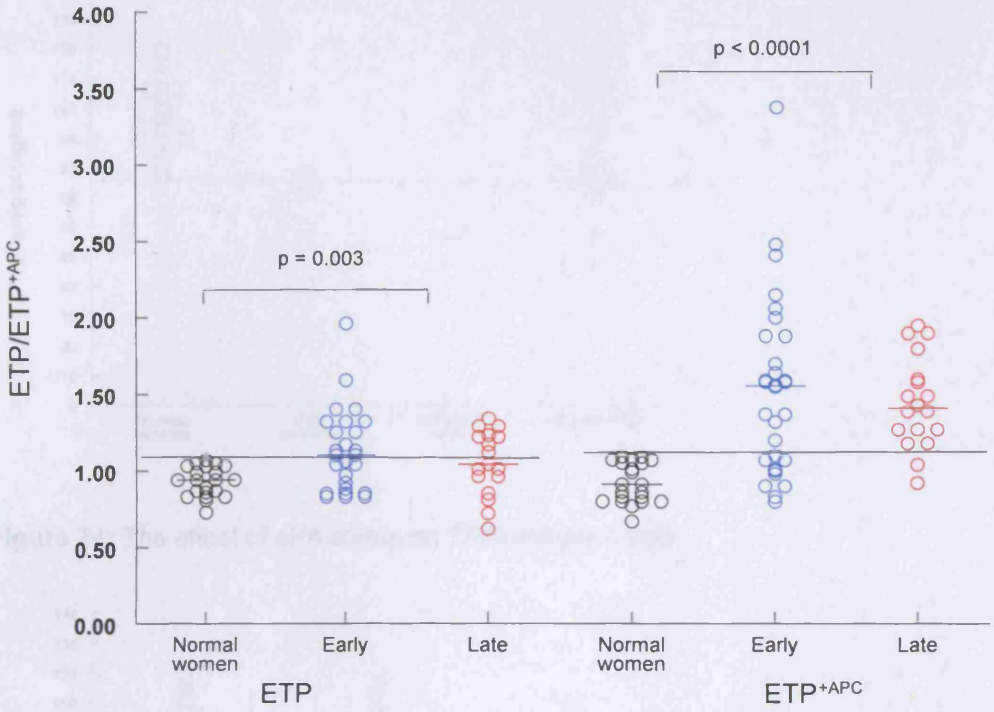

Figure 73: The effect of time of adverse pregnancy event on thrombin generation

7.3.2 TFPI antigen in pregnancy morbidity

A range of 75 – 120 ng/ml was established for TFPI antigen in 23 normal healthy women, of whom none were receiving anticoagulation or oral contraceptives. I was able to study TFPI antigen in 28 of the women with previous pregnancy morbidity. No significant differences were detected in association with aPA status (Figure 74) and time of pregnancy morbidity (Figure 75). However, the numbers of aPA positive and late pregnancy morbidity were relatively small and the precludes any firm conclusions about lack of association. Nevertheless, 12/28 (43%) of women with previous pregnancy morbidity had low TFPI antigen, and the median TFPI antigen was significantly lower in these women than in normals (78 ng/mL vs. 96 ng/mL respectively; P = 0.002).

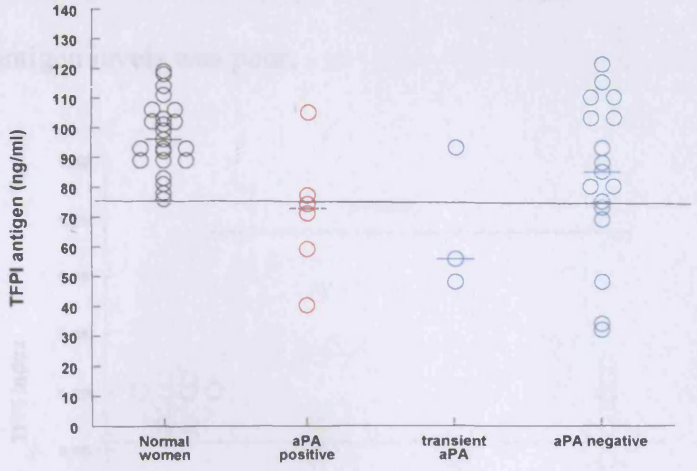

Figure 74: The effect of aPA status on TFPI antigen levels

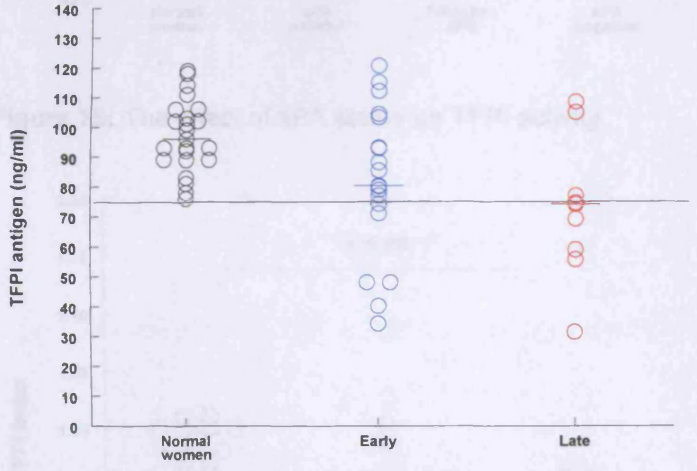

Figure 75: The effect of time of pregnancy morbidity on TFPI antigen levels

7.3.3 TFPI activity in pregnancy loss

TFPI sensitivity index, which is a measure of functional activity, was measured in 20 normal healthy women, giving a 95% confidence range of 0.962 – 1.011 (median 0.987). I studied in 33 of the women with previous pregnancy morbidity; recurrent miscarriage (30), intrauterine death (12), preeclampsia (3) and HELLP (3). The median TFPI sensitivity index was significantly lower in previous pregnancy morbidity than in the control group (0.958 v 0.987; p = 0.007) with low values in 20/33 (61%) women. aPA status (Figure 76) and time of morbidity (Figure 77) had no significant effect. As

with a previous study (Dahm et al 2005), the correlation between TFPI activity and antigen levels was poor.

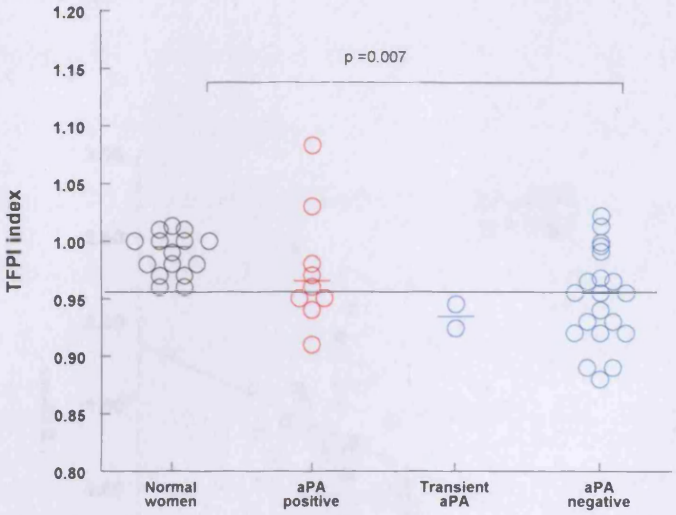

Figure 76: The effect of aPA status on TFPI activity

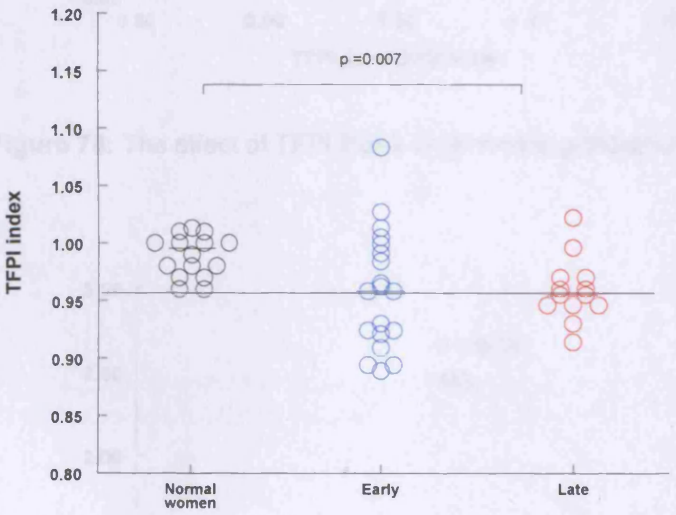

Figure 77: The effect of time of pregnancy morbidity on TFPI activity

A significant inverse correlation was observed between TFPI sensitivity index and ETP^{+APC} (Figure 78, but there was no correlation between TFPI sensitivity index and ETP (Figure 79).

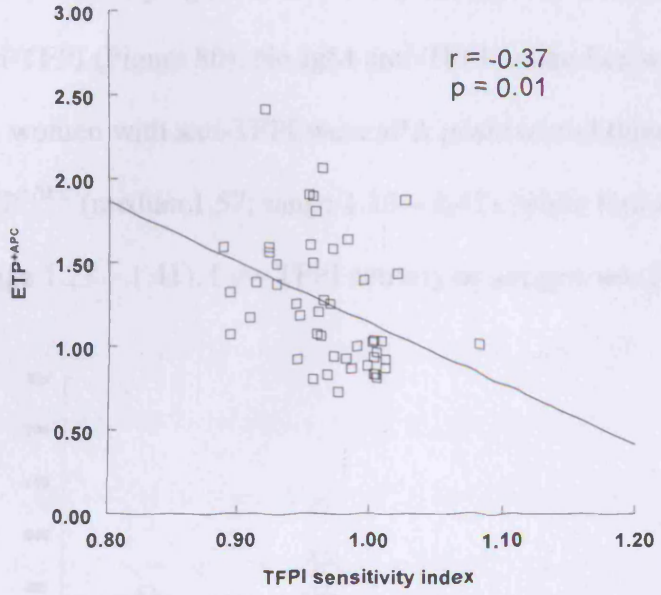

Figure 78: The effect of TFPI index on thrombin generation in the presence of APC

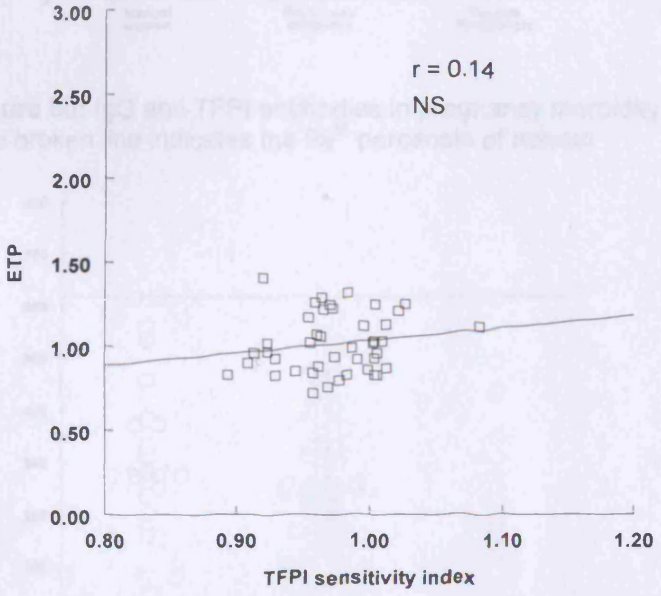

Figure 79: The effect of TFPI index on thrombin generation without APC

7.3.3 The prevalence of anti-TFPI antibodies

I investigated the presence of anti-TFPI antibodies in 48 women with previous pregnancy morbidity and twelve patients with a history of thrombosis and potent aPA. Normality was defined by the 99th percentile of 24 normal healthy women. Six women with previous pregnancy morbidity and two APS thrombosis patients had low titre IgG anti-TFPI (Figure 80). No IgM anti-TFPI antibodies were detected (Figure 81). Three of the women with anti-TFPI were aPA positive and three were aPA negative. All had high ETP^{+APC} (median 1.57; range 1.10 – 2.41), while four had raised ETP (median 1.34; range 1.23 – 1.41). Low TFPI activity or antigen was found in 4/6women.

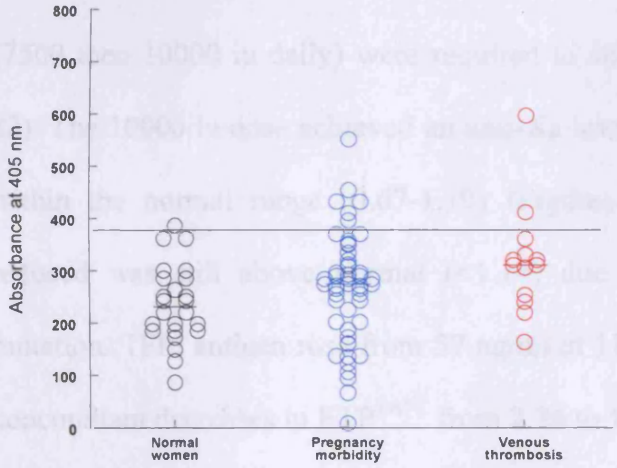

Figure 80: IgG anti-TFPI antibodies in pregnancy morbidity and thrombosis. The broken line indicates the 99th percentile of normal

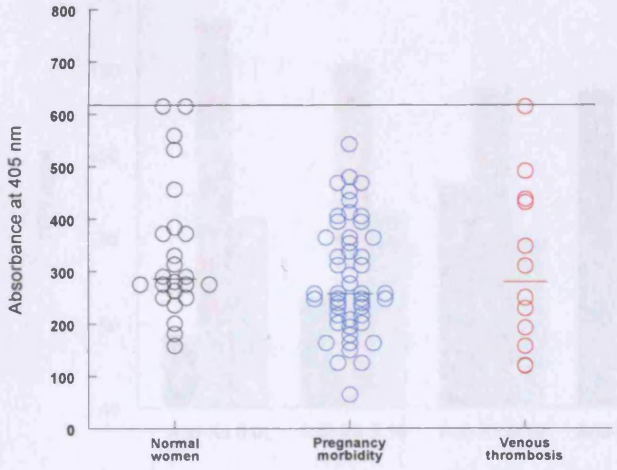

Figure 81: IgM anti-TFPI antibodies in pregnancy morbidity and thrombosis The broken line indicates the 99th percentile of normal

7.3.4 The effect of low molecular weight heparin in a high risk pregnancy

A 32-year-old woman (not previously studied) with a family history of VTE and homozygous for factor V Leiden, was seen in our miscarriage clinic in the 11th week of pregnancy. All tests for aPA were negative, protein C and antithrombin were within normal limits, free protein S was borderline (0.55 IU/ml, normal 0.55 -1.30 IU/ml) and TFPI antigen was low (57.1 ng/ml). At this time she was not receiving anticoagulants, so in accordance with local practice for high-risk pregnancies, daily LMWH (5000 iu Dalteparin) and aspirin were commenced. Anti-Xa levels, TFPI antigen, ETP and ETP^{+APC} were studied serially in an effort to elucidate the relationship between these parameters. She was unusually resistant to LMWH treatment and two dose escalations (7500 then 10000 iu daily) were required to achieve the desired anti-Xa level (Figure 82). The 10000 iu dose achieved an anti-Xa level of 0.44 and brought the ETP value to within the normal range (0.67-1.19) (Figures 83 and 84). The ETP^{+APC}, although reduced was still above normal (<1.14) due to the homozygous factor V Leiden mutation. TFPI antigen rose from 57 ng/ml at 11 weeks to 115 ng/ml at 22 weeks, with concomitant decreases in ETP^{+APC} from 2.76 to 1.80 and ETP from 1.29 to 1.00.

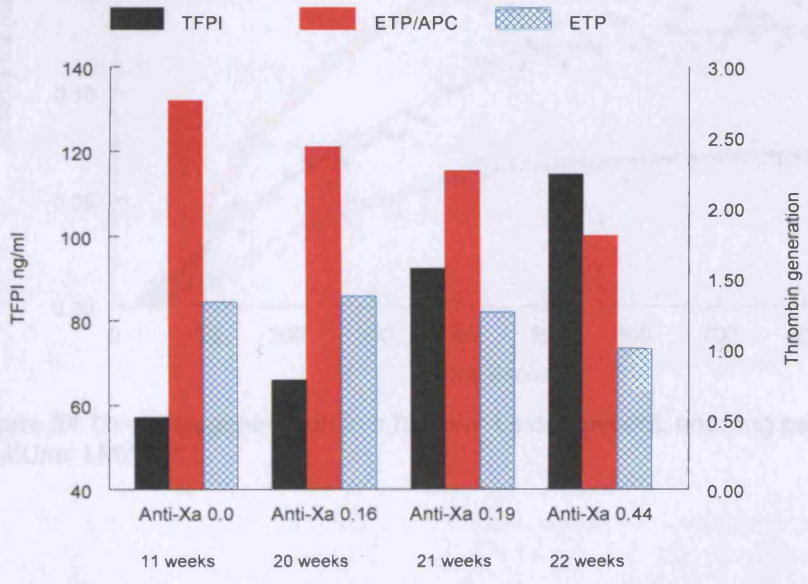

Figure 82 Anti-Xa, TFPI and thrombin generation values in a single high-risk pregnancy.

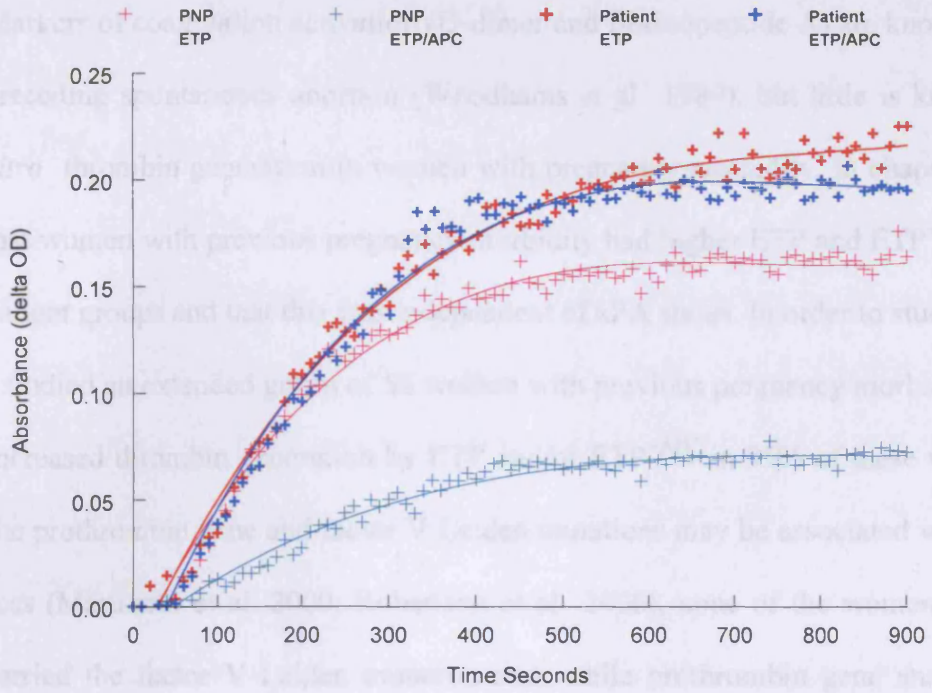

Figure 83 Thrombin generation in a factor V Leiden patient, pre-treatment

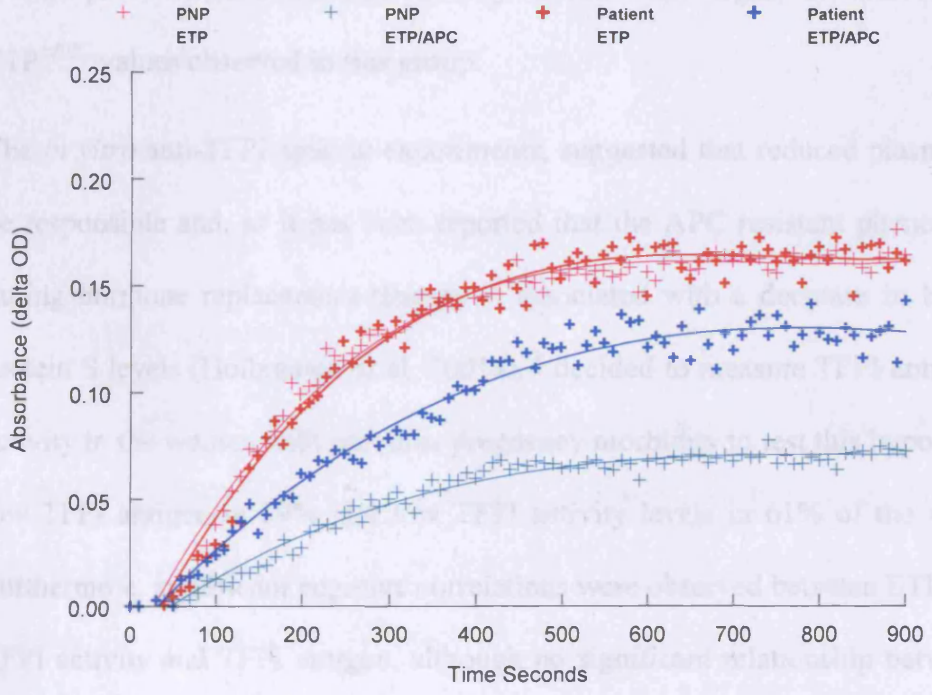

Figure 84 Thrombin generation in a factor V Leiden patient, showing partial normalisation with 0.44IU/ml LMWH

7.4 Discussion

Markers of coagulation activation (D-dimer and fibrinopeptide A) are known to increase preceding spontaneous abortion (Woodhams et al 1989), but little is known about *in vitro* thrombin generation in women with pregnancy morbidity. In chapter 6 I showed that women with previous pregnancy morbidity had higher ETP and ETP^{+APC} than other patient groups and that this was independent of aPA status. In order to study this further, I studied an extended group of 52 women with previous pregnancy morbidity and found increased thrombin generation by ETP and/or ETP^{+APC} in 85% of these women. While the prothrombin gene and factor V Leiden mutations may be associated with pregnancy loss (Martinelli et al. 2000; Robertson et al 2006), none of the women in this group carried the factor V Leiden mutation and, while prothrombin gene analysis was not performed on most of these women, my data from chapter 4 suggested that increased plasma prothrombin alone was unlikely to cause the degree of increase in ETP and ETP^{+APC} values observed in this group.

The *in vitro* anti-TFPI spiking experiments, suggested that reduced plasma TFPI could be responsible and, as it has been reported that the APC resistant phenotype observed during hormone replacement therapy is associated with a decrease in both TFPI and protein S levels (Hoibraaten et al. 2001a), I decided to measure TFPI antigen and TFPI activity in the women with previous pregnancy morbidity to test this hypothesis. I found low TFPI antigen in 59% and low TFPI activity levels in 61% of the women tested. Furthermore, significant negative correlations were observed between ETP^{+APC} and both TFPI activity and TFPI antigen, although no significant relationship between ETP and TFPI was observed.

LMWH has been the mainstay of antithrombotic therapy in women with aPA or hereditary thrombophilia for many years and, although it has is reported to be "the superior choice", on the grounds of safety and effectiveness, in improving pregnancy

outcome (Robertson, Wu, & Greer 2004), the use of LMWH in this setting is still highly controversial (Walker et al. 2005). It has been shown that in a purified system, TFPI and APC act synergistically in the regulation of thrombin generation, and that reduced levels of TFPI exacerbated the APC resistance produced by the factor V Leiden mutation (van 't Veer et al 1997a). However, this work was performed in the presence of saturating concentrations of phospholipid, and the absence of protein S. As protein S is now known to be a cofactor for TFPI in the inactivation of factor Xa (Hackeng et al 2006), and saturating levels of phospholipid are seldom seen *in vivo,* I was unsure if these mechanisms would apply to patient plasma in the presence of limited quantities of phospholipid.

My data show how combined thrombophilic defects (homozygous FVL, low TFPI and protein S) act synergistically to produce almost complete resistance to APC, as demonstrated by increased *in vitro* thrombin generation. The administration of subcutaneous LMWH produced the following effects:

- An increase in plasma TFPI
- A 25% reduction in ETP to the same level as that of pooled normal plasma.
- A 36% decrease in ETP^{+APC}

This is in keeping with my findings in chapter 4, in which I demonstrated that the addition of increasing amounts of a polyclonal antibody, that blocked TFPI function, caused a dose dependent increase in ETP^{+APC} of up to 50% and but only a modest increase in ETP (<10%). Furthermore, I have demonstrated a negative association between plasma TFPI concentration and APC resistance in a range of patients. I have also demonstrated that the restoration of TFPI levels by treatment with LMWH, overcomes APC resistance. These data strongly suggest that deficiency of TFPI produces an APC resistant phenotype, most probably due a failure of TFPI to limit the

initial rate of thrombin production through FXa and hence FVa and FVIIIa production. Unfortunately, due to plasma sample volume constraints, it was not possible to determine the relative importance of TFPI and the anti-Xa/antithrombin effects of LMWH.

It has previously reported that the majority of known thrombophilic defects in women with pregnancy loss are in the protein C pathway. In one study, resistance to APC was been demonstrated in 70% of all women with recurrent pregnancy loss using the Pro-C Global test (Dade-Behring), 52% without a known thrombophilic risk factor (Sarig et al 2002). However, the antiphospholipid status of these patients was not investigated in this study and, as discussed in chapter 3, I have shown this test to be susceptible to interference by lupus anticoagulant (Gardiner et al. 2002).

The relationship between APC resistance and pregnancy morbidity is disputed. This may be in part because the ETP based assay and the two types of APTT-based APC sensitivity tests (with and without predilution in factor V deficient plasma) have different determinants (de Visser et al 2005). Many cases of unexplained pregnancy loss occur in the absence of aPA or known thrombophilic defects, but is known that many pregnant women develop a physiological resistance to APC during pregnancy (Cumming et al. 1995) and that acquired APC resistance (measured by the APTT based method), independent of the factor V Leiden mutation (FVL), is a risk factor for pregnancy loss (Rai et al. 2001; Robertson et al 2006), preeclampsia (Paternoster et al. 2002) and low birth weight (Clark et al. 2001) .

Mercier *et al* (1998) reported that anti-β_2GPI from women suffering recurrent miscarriages caused APC resistance. It has also been reported that anti-β_2GPI interfere with the phospholipid-dependent inhibition of TF-induced coagulation by TFPI (Salemink et al. 2000). However, although six of the TFPI deficient women in my study had anti-β_2GPI, the majority did not. Anti-TFPI antibodies have also been reported in

women with aPA and recurrent miscarriage (Martinuzzo et al. 2005), but I found anti-TFPI in only 6/48 women studied with previous pregnancy loss. I concluded that, in the majority of cases, there is little evidence for an antibody dependent mechanism for TFPI deficiency in women with previous pregnancy morbidity.

It is clear that many haemostatic mechanisms may contribute towards recurrent pregnancy loss and preeclampsia. Many of these may be antibody mediated: aPA binding to normal term placenta (Donohoe, Kingdom, & Mackie 1999); antibodies to annexin V (Gris et al. 2000; Rand et al 1997); antibodies to EPCR (Hurtado et al 2004); and antibodies directed against factor XII (Jones et al 2001). In addition to APC resistance, other antibody-independent haemostatic defects have been implicated in pregnancy morbidity (Preston et al 1996): hyperfibrinolysis and low factor XII (Gris et al. 1997), increased factor VIII:c levels (Dossenbach-Glaninger et al. 2004; Marietta et al. 2003) although this had been disputed (Middeldorp et al. 2004); the prothrombin gene and factor V Leiden mutations (Martinelli et al 2000); reduced placental TFPI (Aharon et al. 2005; Sarig et al 2005); and protein S deficiency(Sarig et al 2002)

Treatment of pregnant women who have aPA and recurrent pregnancy loss with unfractionated heparin and aspirin (Rai et al 1997) aspirin (Farquharson et al 2002) and/or low molecular weight heparin is reported to improve the live birth rate (Backos et al 1999; Brenner et al 2005; Gris et al. 2004) although the precise mechanisms for the protective effect of heparin in this setting are unclear. Although it was often assumed that it was the anticoagulant effect of LMWH that afforded this protection, significant activation of the coagulation system still occurs during LMWH treatment (Hoke et al. 2004)and several other mechanisms have been proposed. Heparin has been shown to attenuate placental apoptosis (Bose et al. 2005), prevent complement activation on decidual cells (Girardi, Redecha, & Salmon 2004) and prevent binding of aPA (McIntyre et al. 1993) .

Treatment of recurrent pregnancy loss associated with other thrombophilic defects is a hotly debated topic, which is unlikely to be resolved until further randomised controlled trials are performed (Walker et al 2005). Despite this treatment with low molecular weight heparin has been used in the treatment of recurrent pregnancy loss associated with thrombophilia for several years. It is known that placental TFPI, which may be decreased in gestational vascular complications, can be restored by maternal LMWH treatment (Aharon et al 2005; Sarig et al 2005).

My data suggest that TFPI deficiency associated with ETP dependent APC resistance could be a risk factor for pregnancy loss, and imply a potential role for heparin in the treatment of this condition.

Chapter 8. Discussion

There is a great deal of heterogeneity in the specificity, concentration and avidity of aPA and not all of the target antigens are represented by the antibodies detected by the typical clinical laboratory. Furthermore, there is poor standardisation of assays for aPA and a lack of agreement over their pathological mechanisms. It was against this background of that I hypothesised that a global measurement of the coagulation and protein C anticoagulant pathways might be more informative than the traditional immunological markers. In this thesis I have studied the phenomenon of acquired resistance to protein C and its association with the antiphospholipid syndrome.

In chapter 3 I showed that plasma from some patients with APS interfere with the protein C pathway and that this may manifest as APC resistance in the absence of the factor V Leiden mutation. These preliminary results suggested that the antibodies which caused LA activity, and those that interfered with the protein C pathway, were not always found in the same patients, and that their detection was highly dependent upon the reagents/method used.

In the fourth chapter I described how I developed method for measuring the anticoagulant response to APC, which was more sensitive to aPA, so that I could investigate my initial findings further. I confirmed the importance of zwitterionic phospholipids for APC function. Using this method, I found resistance to APC in the majority of the patients with APS studied and I also showed that this occurred in an antibody dependent manner. The data also suggested that while most patients with anti-β_2GPI demonstrated resistance to APC as previously reported (Martinuzzo et al 1996), the converse was not true, in that most patients with APC resistance did not have anti-β_2GPI.

In chapter 5, I described the development and characterisation of a thrombin generation based method to measure sensitivity to APC. The preliminary data suggested that in the majority of patients with aPA, thrombin generation was abnormal, with a variable anticoagulant response to APC. In demonstrating a delay in thrombin generation coupled with a failure of APC to inhibit thrombin generation, I have established a potential mode of action for lupus anticoagulants, which could explain both the observed prolonged *in vitro* clotting time and the paradoxical increase in markers of in vivo thrombin generation and thrombotic risk.

I developed these ideas further in Chapter 6. Using automated thrombin generation to study a much larger cohort of patients, I found that the thrombin generation and sensitivity to APC were highly heterogeneous. While aPA status had no significant effect of ETP (in the absence of APC), ETP^{+APC} was significantly higher in aPA patients with thrombosis than in aPA negative thrombosis patients. However, women with previous pregnancy morbidity significantly higher ETP and ETP^{+APC} values than any other group of patients studied, and this was independent of aPA status. I demonstrated that a failure of APC to effectively downregulate thrombin generation is associated with LA and, while this may be due in part to anti-β_2GPI antibodies, β_2GPI independent mechanisms also exist.

In chapter 7, I went on to study the excess thrombin generation in a larger group of women with a history of pregnancy morbidity. Increased thrombin generation with and without APC were found in the majority of women with a history of pregnancy morbidity and approximately half of the women with previous pregnancy morbidity had low TFPI antigen and/or low TFPI activity. Low titre anti-TFPI antibodies were found in only a few of these women and aPA status had no significant effect on thrombin generation, or TFPI levels. These findings occurred in the non-pregnant state, indicating an underlying prothrombotic condition. I demonstrated in one woman that treatment

with LMWH restored TFPI antigen levels to normal with a concomitant decrease in ETP^{+APC}. My data suggested that TFPI deficiency associated with ETP dependent APC resistance could be a risk factor for pregnancy loss, and implied a potential role for heparin in the treatment of this condition.

In developing the methods used in this thesis, I have been mindful of the necessity of achieving the correct balance in terms of sensitivity to the different coagulant and anticoagulant pathways. At very low tissue factor concentrations, thrombin generation is exquisitely sensitive to TFPI and procoagulant pathways, but less sensitive changes in the protein C pathway (Baglin 2005). I wanted to assess the response to APC while retaining sensitivity to TFPI and factor VIIIc levels. This will always be a compromise and I am fully aware no single thrombin generation method will be suitable for studying all bleeding and thrombotic conditions. The method that I have used, does not take into account the important roles that platelets and fibrinogen/fibrin play in thrombin generation (Baglin 2005) and I realise that this is a serious limitation of this work. However, I believe that I have achieved an acceptable compromise through which I have been able to simultaneously assess TFPI, the response to APC, and prothrombotic conditions such as the acute phase response.

8.1 Can activated protein C resistance be used to diagnose antiphospholipid syndrome

I have demonstrated APC resistance by different methods in a significant proportion of patients with APS. However, the APC resistance phenotype is far from specific for the presence of aPA, with factor VIII, protein S, prothrombin and TFPI all acting as confounding variables. Although this study was not sufficiently powered to test for an association between APC resistance and the severity of disease, many of the patients with the most severe forms of APS had very high ETP^{+APC} values. This work was

performed on retrospective cohorts, rather than in a prospective fashion on consecutive patients and this is a limitation of this study.

However, I have found significant associations between increased thrombin generation/APC resistance and the presence of aPA in patients with thrombosis. I believe that prospective studies are now warranted to determine whether APC resistance could be clinically useful in the diagnosis and management of APS.

8.2 Can interference in the protein C pathway explain the thrombogenicity of antiphospholipid antibodies

Since the inception of this work there have been some significant advances in our knowledge of the protein C system. At that time, the prevailing view was that only β_2GP-I dependent antibodies were responsible for aPA associated APC resistance (Galli et al 1998; Martinuzzo et al 1996; Mercier et al 1998; Viveros et al 2005). Much of this work was based on differentiation of anti-prothrombin from β_2GPI dependent antibodies by means of the KCT and dRVVT tests. However, this was based on the ability of cardiolipin vesicles to adsorb aPA, rather than a genuine demonstration of anti prothrombin and or anti-β_2GPI activity and the conclusions were drawn from results from relatively few patients. These data were extrapolated to support the claim that only β2GPI dependent aPA were responsible for the inhibition of APC (Galli et al 1998), a view that this group now concede was incorrect (Galli et al 2005). Other publications relied on the use of monoclonal antibodies (Hwang et al 2001; Hwang et al 2003) which do not necessarily act as one would expect in biological systems.

I have demonstrated in several systems that aPA associated resistance to APC may be antibody mediated. Furthermore I have shown that this may occur through both β_2GPI dependent and β_2GPI independent mechanisms. However, not all of the immunoglobulin fractions that I purified from aPA patients caused resistance to APC

when added to normal plasma. It is likely that aPA dependent resistance to APC is only one of the many thrombogenic mechanisms present in APS.

I have confirmed the presence of antibodies to TFPI in a small number of patients and although I cannot discount an autoimmune mechanism for TFPI deficiency, it is unlikely that this alone can explain the high incidence of APC resistance and increased thrombin generation that I have described in women with a history of pregnancy morbidity. It is clear that the causes of acquired APC resistance are complex. The APC resistant phenotype observed during hormone replacement therapy is associated with a decrease in both TFPI and protein S levels (Hoibraaten et al. 2001b). Furthermore, it has recently been reported that protein S may act as a cofactor for the TFPI dependent inactivation of factor Xa (Hackeng et al 2006), and it was not until then that I fully understood the complex relationship between TFPI and the protein C system. This supports the "threshold limited" hypothesis of thrombin generation (van 't Veer et al 1997b), in which TFPI limits the rate of factor V activation, thus allowing the regulation of prothrombinase activity by APC. When TFPI concentration is low, the rapid rate of FVa formation swamps APC, whereas when TFPI levels are sufficiently high, even the limited proteolysis of activated factor V Leiden by APC has an appreciable effect on thrombin generation. I have demonstrated, albeit in one patient, that LMWH can partially attenuate the factor V Leiden phenotype through increasing TFPI levels. I believe that this may explain the reported efficacy of LMWH treatment in patients with the factor V Leiden mutation in high-risk situations.

8.3 Future directions

It has been known for some time that platelet microparticles have procoagulant activity (Berckmans et al. 2001; Nieuwland et al. 1997; Tans et al. 1991) and may produce an APC resistant phenotype (Taube et al 1999). Microparticle shedding from the placenta into the maternal circulation has also been implicated in the pathology of preeclampsia

(Goswami et al. 2006), and this in association with APC resistance combined has been suggested as a possible mechanism for coagulation activation in this disease (VanWijk et al. 2002). Recently, an association between circulating platelet-derived microparticles, thrombin generation and a procoagulant state has been reported in systemic lupus erythematosus (Pereira et al. 2006).

Against this background, it is not surprising that microparticles have been implicated in the antiphospholipid syndrome (Ambrozic et al. 2005; Dignat-George et al. 2004; Morel et al. 2005). While I was not wholly unaware of this during the course of my work, the technology to measure, identify, purify and remove microparticles was not easily obtainable. With hindsight, I would liked to have studied the role of microparticles from various cell types on the pathology of APS, their role in thrombin generation and resistance to APC resistance and I hope to study these areas in the near future.

Publications arising from this thesis

Papers

C Gardiner, SJ Machin, IJ Mackie (2007) Measuring thrombin generation based sensitivity to activated protein C using an automated coagulometer (ACL 9000). Accepted for publication in *The International Journal of Laboratory Hematology*

C Gardiner, H Cohen, SK Austin, SJ Machin, IJ Mackie (2006) Pregnancy loss, TFPI deficiency and resistance to activated protein C. *Journal of Thrombosis and Haemostasis* 4: 2724-6

C Gardiner, H Cohen, AH Jenkins, SJ Machin, IJ Mackie (2006) Detection of acquired resistance to activated protein C associated with antiphospholipid antibodies using a novel clotting assay. *Blood Coagulation and Fibrinolysis* **17**: 477-483

C. Gardiner, P.C. Cooper, M. Makris, IJ Mackie, R.G. Malia and SJ Machin (2002) An evaluation of screening tests for defects in the protein C pathway: commercial kits lack sensitivity and specificity. *Blood Coagulation & Fibrinolysis* **13**:155-163

Abstracts

C Gardiner, SJ Machin, IJ Mackie (2007) Evidence for $\beta_2$glycoprotein independent antiphospholipid-associated activated protein C resistance. BSHT 2007

C Gardiner, H. Cohen, SJ Machin, IJ. Mackie (2007) Pregnancy morbidity is associated with low plasma tissue factor pathway inhibitor levels, increased thrombin generation and resistance to activated protein C *J Thrombosis and Haemost*; **5,** S2. Abstract No. P-M-600

C Gardiner, H Cohen, SJ Machin, IJ Mackie (2006) Recurrent pregnancy loss, TFPI deficiency and resistance to activated protein C. *Platelets* **17**: Suppl 1; S10

C Gardiner, H Cohen, AH Jenkins, SJ Machin, IJ Mackie (2006) Why do antiphospholipid antibodies cause Thrombosis and Pregnancy Loss? *Laboratory Hematology* **12**: 161

C Gardiner, IJ Mackie, H Cohen, SJ Machin (2005) Measuring the Haemostatic Phenotype of the Antiphospholipid Syndrome. Presented at the BSHT annual scientific meeting at Chester September 2005.

C Gardiner, IJ Mackie, K Piegsa, SA Furs, J Guillebaud, SJ Machin. (2003) Activated protein C resistance (APCR) and combined oral contraceptives: acquired APCR is more pronounced in women receiving 3rd generation COCs than those containing levonorgestrel and is associated with low protein S levels. *J Thrombosis and Haemost*; 1 Supplement 1 Abstract number: P0883d

C Gardiner, I J Mackie, K Piegsa, SA Furs., J Guillebaud., SJ Machin (2003) Acquired resistance to activated protein C is more pronounced in combined oral contraceptives containing desogestrel than levonorgestrel. *Br J Haematology* 121 Supplement 1, 62

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